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The International Congress of Toxicology-VII was held July 2-6, 1995, at the Washington State Convention Center, Seattle. The theme of the Congress was *Horizons in Toxicology: Preparing for the 21st Century*. There were nearly 2500 attendees from over 50 countries, including 1878 scientific registrants, 428 exhibitor registrants, 177 guests, and 8 media representatives. The scientific presentations of the Congress included the Deichmann lecture, Keynote lecture, 4 plenary lectures, 21 symposia, 13 workshops, 6 continuing education courses (4 offered twice) with 771 attendees, 2 debates, and 1331 abstracts for posters, poster discussions, and short oral communication presentations. The Congress proceedings appeared as a special issue of *Toxicology Letters*, Vol. 82/83 (1995), and also as a bound volume. The 994-page volume comprises 136 papers.

The Congress was viewed as a success by all attendees. The presentations were of exceptional scientific quality and provided both a comprehensive review of current knowledge in toxicology and consideration of the prospects for the acquisition of new knowledge. The presentations reported on information obtained from studies at the molecular and cellular levels up to intact animals and, indeed, populations of laboratory animals and humans. Substantial emphasis was given to the application of toxicological information to the assessment of human health risks. The Congress received support from 50 industrial organizations, professional societies, and government agencies and involved thousands of hours of volunteer effort.

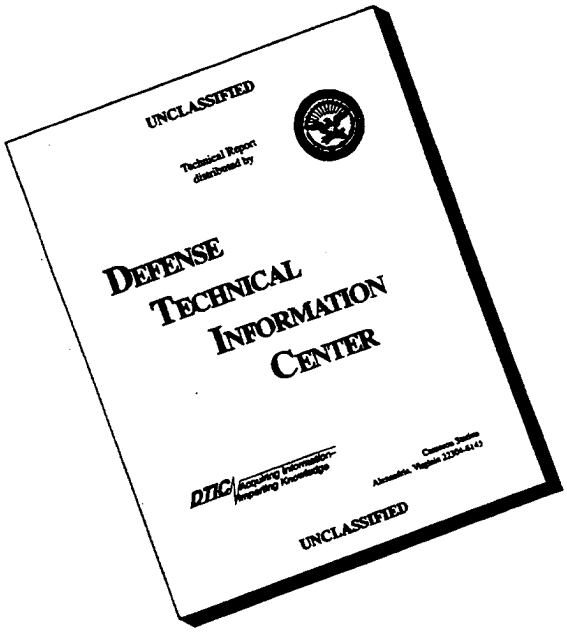
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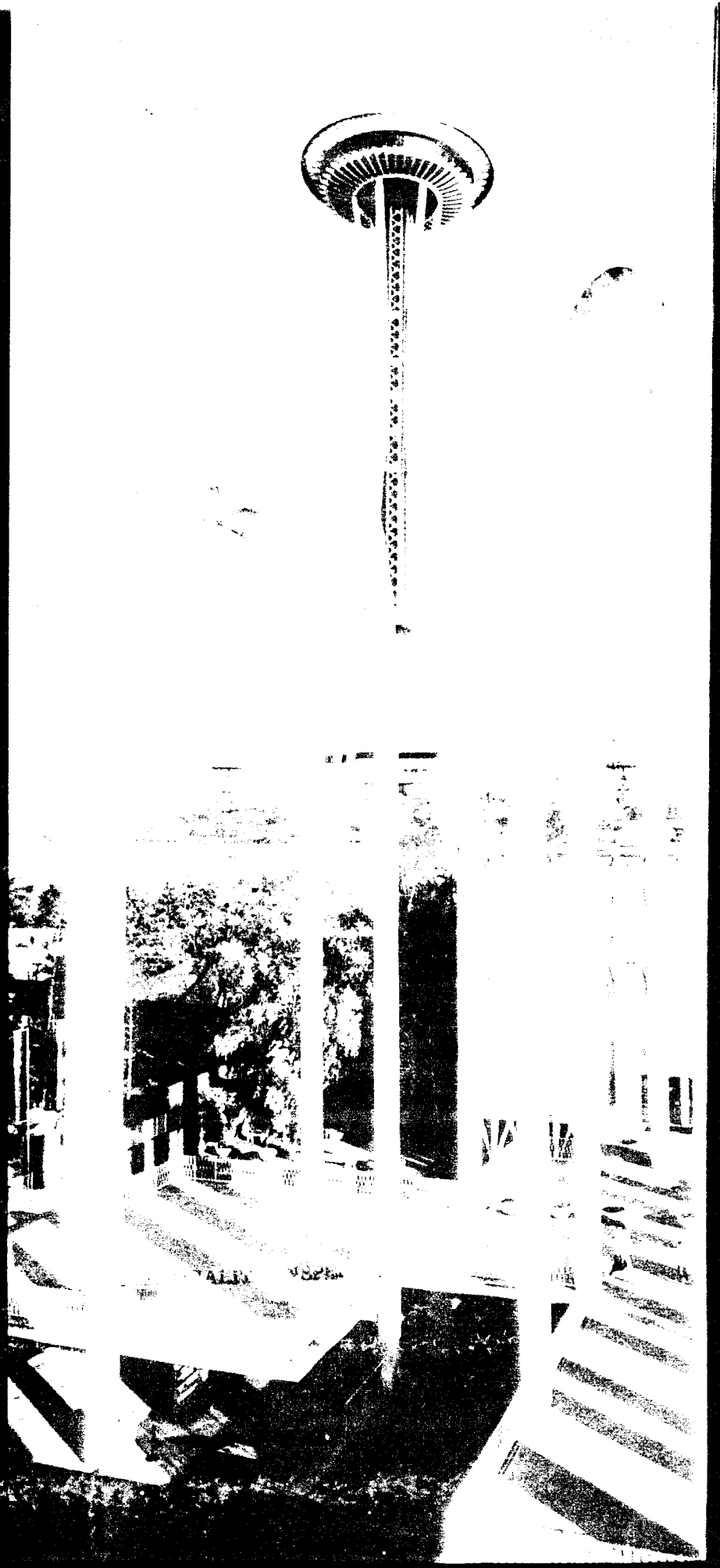
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Toxicology Letters

Preface

The Seventh International Congress of Toxicology was held in Seattle, Washington, USA from July 2 to July 6, 1995. Over 2000 scientists from more than 50 countries participated in scientific sessions and social events, and viewed exhibits provided by more than 100 exhibitors. The theme of the Congress was 'Horizons in Toxicology: Preparing for the 21st Century'. The scientific presentations of the Congress included the prestigious Deichmann Lecture, Keynote Lecture, 4 Plenary Lectures, 21 Symposia, 13 Workshops, 6 Continuing Education Courses (four of which were offered twice), 2 Debates and 1331 abstracts for Posters, Poster Discussions, and Short Oral Communications.

To insure rapid publication of the proceedings, an Editorial Review Committee consisting of the following members provided reviews during the Congress of a majority of the submitted manuscripts.

Chantel Bismuth	James Bus
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Ernest Hodgson	Steven Gilbert
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Paolo Preziosi	Lewis Smith
Ken Wallace	

The administrative tasks associated with the manuscript reviews were performed in very able fashion by Roxanne Bodine and Carolyn Knapp.

Many thanks go to all of those who were so willing to assist in the publication of these proceedings. Financial support for the Congress is also acknowledged; contributions range from the major contributors shown here to those attendees who in part or entirely paid the costs of attending the Congress. Without these sacrifices the gathering of scientists from around the world would not have been possible.

Toxicology is a rapidly advancing field of scientific endeavour with a multidisciplinary base that gives a unique quality to the research that ranges from epidemiology to the mechanistic basis for the understanding of toxic events. This volume reflects such a range of scientific research and truly represents the theme of the Congress. It has been a pleasure to have the opportunity to read each manuscript and to enjoy the excitement and fervor of toxicology today with a focus on the coming Century. We look forward to the Eighth International Congress of Toxicology to be held in 1998 in Paris.

Donald J. Reed
Corvallis, Oregon
August 31, 1995

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1995 Deichmann Lecture—p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology and cancer risk assessment

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Abstract

Carcinogenesis is a multistage process involving activation of protooncogenes, e.g., ras, and inactivation of tumor suppressor genes, e.g., p53 and $p16^{INK4}$. p53 is a prototype tumor suppressor gene that is well suited for analysis of mutational spectrum in human cancers; it is the most common genetic lesion in human cancers, it is a reasonable size for a molecular target, and it may indicate selection of mutations with pathobiological significance. The p53 mutational spectrum differs among cancers of the colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues and hemopoietic tissues. Analysis of these mutations can provide clues to the etiology of these diverse tumors and to the function of specific regions of p53. Transitions predominate in colon, brain and lymphoid malignancies. Mutational hotspots at CpG dinucleotides in codons 175, 245, 248, 273 and 282 may reflect endogenous mutagenic mechanisms, e.g., deamination of 5-methylcytosine to thymidine. Oxy-radicals including nitric oxide may enhance the rate of deamination. G:C to T:A transversions are the most frequent substitutions observed in cancers of the lung, breast, esophagus and liver, and are more likely to be due to bulky carcinogen-DNA adducts. G to T transversion is more common in lung cancers from smokers when compared to never smokers. The high frequency of p53 mutations in the nontranscribed DNA strand is a reflection of strand specific repair. p53 mutation and/or accumulation of p53 protein can be preinvasive events in bronchial or esophageal carcinogenesis. p53 mutations also generally indicate a poor prognosis. In geographic areas where hepatitis B virus (HBV) and aflatoxin B_1 are cancer risk factors, most mutations are at the third nucleotide pair of codon 249. In geographic areas where hepatitis B and C virsus – but not aflatoxin B_1 – are risk factors, the p53 mutations are distributed in numerous codons. HBV X protein complexes with the p53 protein and inhibits its sequence specific DNA binding, transactivating and apoptotic capacity. The mutation load of 249^{ser} mutant cells in nontumorous liver is positively correlated with dietary aflatoxin B_1 exposure. The induction of skin carcinoma by ultraviolet light is indicated by the occurrence of p53 mutations at dipyrimidine sites including CC to TT double base changes. In summary, these differences in mutational frequency and spectrum among human cancer types suggest the etiological contributions in both exogenous and endogenous factors to human carcinogenesis and have implications for human cancer risk assessment.

Keywords: p53 tumor suppressor gene; Carcinogenesis; Cancer risk assessment; Mutagenesis

The crucial differences between normal and cancer cells stem from discrete changes in specific genes controlling proliferation and tissue homeostasis. Over 100 such cancer-related genes have been discovered, several of which are implicated in the natural history of human cancer because they are consistently found mutated in tumors. The p53 tumor suppressor gene is the

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Table 1
Examples of tumor suppressor genes involved in human cancers

Tumor suppressor gene	Chromosomal locus	Location/ proposed function	Somatic mutations		Inherited mutations		
			Major types of somatic mutation	Examples of neoplasms with somatic mutations	Syndrome	Heterozygote carrier rate 10^5 births	Typical neoplasms
p53	17p13.1	Nuclear/ transcription factor	Missense	Most human cancer types examined to date	Li-Fraumeni	~2	Carcinomas of breast and adrenal cortex; sarcomas; leukemia; brain tumors
RB1	13q14	Nuclear/ transcription modifier	Deletion and nonsense	Retinoblastoma; osteosarcoma; carcinomas of the breast, prostate, bladder, and lung	Retinoblastoma	~2	Retinoblastoma; osteosarcoma
APC	5q21	Cytoplasmic/ unknown	Deletion and nonsense	Carcinoma of the colon, stomach and pancreas	Familial adenomatous polyposis	~10	Carcinomas of colon, thyroid and stomach
ATM	11q22	Unknown/kinase	Deletion	Unknown	Ataxia telangiectasia	~2–50	Leukemia; lymphoma
WT1	11p13	Nuclear/ transcription factor	Missense	Wilms' tumor	Wilms' tumor	~0.5–1	Wilms' tumor
NF1	17q11	Cytoplasmic/ GTPase activating protein	Deletion	Schwannomas	Neurofibromatosis type 1	~30	Neural tumors
NF2	22q	Cytoplasmic/ cytoskeletal-membrane linkage	Deletion and nonsense[a]	Schwannomas and meningiomas	Neurofibromatosis type 2	~3	Central schwannomas and meningiomas
$p16^{INK4}$	9p21	Nuclear cyclin dependent kinase inhibitor	Deletion and nonsense	Mesothelioma; pancreas; melanoma; glioblastoma	Familial melanoma ?		Melanoma
VHL	3p25	Nuclear/ adaptor	Deletion[a]	Unknown	von Hippel-Lindau	~3	Hemangioblastoma and renal cell carcinoma

Reviewed in [1,2].
[a] Decreased expression controlled by epigenetic mechanism (DNA methylation) [3].

most striking example because it is mutated in about half of almost all cancer types arising from a wide spectrum of tissues. Other tumor suppressor genes important in human oncology, e.g., APC, WT1, $p16^{INK4}$ or NF1 may have a more limited distribution (Table 1), but given the variety of hereditary cancers and allelic deletions found in human cancers, additional tumor suppressor genes should be identified in the future, some of which may also have a conspicuous role in carcinogenesis.

Tumor suppressor genes are vulnerable sites for critical DNA damage because normally they function as physiological barriers against clonal expansion or genomic mutability, and are able to hinder growth and metastasis of cells driven to uncontrolled proliferation by oncogenes. Loss of tumor suppressor function can occur by damage to the genome through mutation, chromosomal rearrangement and nondisjunction, gene conversion, imprinting, or mitotic recombination. Tumor suppressor activity also can be neutralized by interaction with other cellular proteins or with viral oncoproteins. Comprehensive reviews of this rapidly advancing field of molecular carcinogenesis are available [4–6].

The p53 suppressor gene is the most prominent example because it is mutated in about half of human cancer cases [7,8]. Although the retinoblastoma and APC tumor suppressor genes are most commonly inactivated by nonsense mutations that cause the protein to be truncated or unstable, about 80% of p53 mutations are missense mutations that change the identity of an amino acid. Changing amino acids in this way can alter the protein conformation and increase the stability of p53; it can also alter sequence-specific DNA binding and transcription factor activity of p53 [9]. One explanation for the high frequency of p53 mutation is that the missense class of mutations can cause both a loss of tumor suppressor function and a gain of oncogenic function by changing the repertoire of genes whose expression are controlled by this transcription factor [10,11]. The central role of p53 in multistage carcinogenesis places it at the intellectual crossroads of molecular carcinogenesis, molecular epidemiology of human cancer, and cancer risk assessment.

p53 participates in many cellular functions – cell cycle control, DNA repair, differentiation, genomic plasticity, and programmed cell death [8,10,12]. p53 is one component of the DNA damage response pathway in mammalian cells (Fig. 1). Some of these normal cellular functions of p53 can be modulated and sometimes inhibited by interactions with either cellular proteins, e.g., mdm2, or oncoviral proteins, e.g., hepatitis B virus X protein, of certain DNA viruses. p53 is clearly a component in a biochemical pathway or pathways central to human carcinogenesis, and p53 mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells.

The spectrum of p53 mutations induced in human cancer can help identify particular carcinogens and define the biochemical mechanisms responsible for the genetic lesions in DNA that cause human cancer. The frequency and type of p53 mutations can also act as a molecular dosimeter of carcinogen exposure and thereby provide information about the molecular epidemiology of human cancer risk. The p53 gene is well suited for this form of molecular archaeology. The majority of mutations in p53 are in the hydrophobic midregion of the protein (Fig. 2) [8]. The function of the p53 protein as a transcription factor is exquisitely sensitive to con-

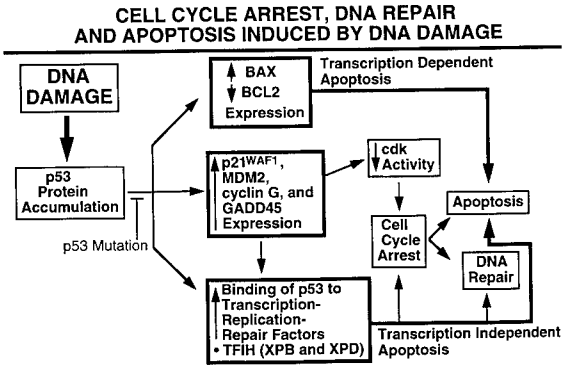


Fig. 1. DNA damage leads to p53 accumulation and subsequent changes in gene expression and protein-protein interactions.

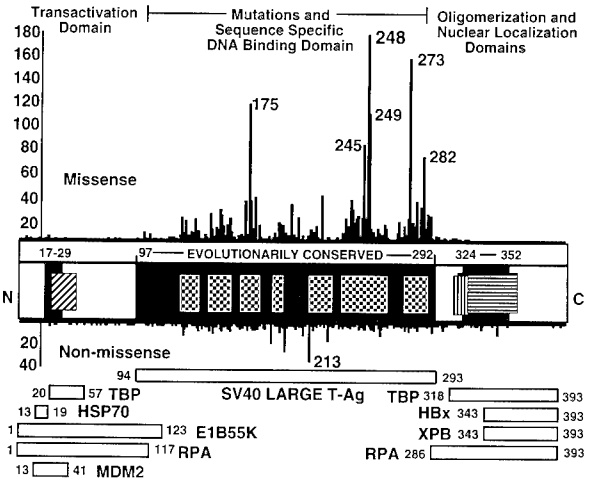


Fig. 2. Schematic representation of p53 molecule. The p53 protein consists of 393 amino acids with functional domains, evolutionarily conserved domains, and regions designated as mutational hotspots. Functional domains include the transactivation region (amino acids 20–42, diagonal striped blocks), sequence-specific DNA binding region (amino acids 100–293), nuclear localization sequence (amino acids 316–325, vertical lined block), and oligomerization region (amino acids 319–360, horizontal striped block). Cellular or oncoviral proteins bind to specific areas of the p53 protein. Evolutionarily conserved domains (amino acids 17–29, 97–292, and 324–352; black areas) were determined using the MACAW program. Seven mutational hotspot regions within the large conserved domain are identified (amino acids 130–142, 151–164, 171–181, 193–200, 213–223, 234–258, and 270–286, checkered blocks). Vertical lines above the schematic, missense mutations; lines below schematic, nonmissense mutations. The majority of missense mutations are in the conserved hydrophobic midregion, while nonmissense (nonsense, frameshift, splicing, and silent mutations) are distributed throughout the protein, determined primarily by sequence context.

formational changes in this region that result from amino acid substitutions [13], and p53 binding to other cellular and oncoviral proteins can easily be disrupted by mutations in this region.

How can p53 mutation spectra lead to identification of the carcinogens that caused a particular tumor? Different carcinogens cause characteristic mutations. Exposure to one common carcinogen, ultraviolet light, is correlated with transition mutations at dipyrimidine sites [14]; dietary aflatoxin B_1 exposure is correlated with G:C to T:A transversions that lead to a serine substitution at residue 249 of p53 in hepatocellular carcinoma [15,16]; and exposure to cigarette smoke is correlated with G:C to T:A transversions in lung carcinomas [17].

How these mutations arise can be further tested in the laboratory. For example, the predominant base changes in p53 found in lung cancers (G:C to T:A transversions) and skin carcinomas (C:G to T:A transitions) suggest that the causal lesion likely occurred on the nontranscribed strand, a finding that is consistent with the preferential repair after damage of the transcribed strand of active genes [18]. Benzo[*a*]pyrene, a carcinogen in tobacco smoke, forms DNA adducts that are more slowly repaired when present on the nontranscribed strand than on the transcribed strand of the hypoxanthine (guanine) phosphoribosyl transferase gene [19], and ultraviolet light-induced crosslinks of dipyrimidines in the nontranscribed DNA strand of the p53 gene also are more slowly repaired than in the transcribed strand [20]. Because DNA repair rates can be sequence-dependent [21], the p53 mutation spectrum could be influenced by both the type and location of the promutagenic lesion. Transcription-repair coupling factors, the products of the mfd, XPB (ERCC-3) and XPD (ERCC-2) genes, have been recently identified and provide a mechanistic underpinning for strand-specific repair [22–24]. The p53 protein binds to XPB and XPD DNA helicases in the TFIIH complex and modulates their function in nucleotide excision repair [25]. Another example comes from areas of China and Mozambique with a high incidence of liver cancer. The high frequency of G:C to T:A transversions in human hepatocellular carcinomas in this region could be due to the high mutability of the third base of codon 249 by aflatoxin B_1 or a selective growth advantage of hepatocyte clones carrying this specific p53 mutant in liver chronically infected with hepatitis B virus. Indeed, the third base of codon 249 in a human liver cell line exposed to aflatoxin B_1 is preferentially mutated [26] and transfected 249^{ser} p53 mutant enhances the growth rate of the p53 null hepatocellular carcinoma cell line, Hep3B [27]. Other p53

codons show a lower frequency of G:C to T:A, G:C to A:T, and G:C to C:G mutations, which suggests that both preferential mutability and clonal selection are involved in human hepatocellular carcinogenesis.

The p53 mutational spectra also can provide molecular evidence that a particular cancer did not result from an environmental carcinogen but instead was caused by endogenous mutagenesis. The high frequency of C to T transitions at CpG dinucleotides in colon carcinomas [7] is consistent with mutagenesis by endogenous deamination mechanisms. A transition of C to T would be generated by spontaneous deamination of 5-methylcytosine [28] or by enzymatic deamination of cytosine by DNA (cytosine-5)-methyl transferase when *S*-adenosylmethionine is in limiting concentration (or by both mechanisms) [29]. Because oxygen radicals enhance the rate of deamination of deoxynucleotides [30,31], chronic inflammation and nitric oxide generated by nitric oxide synthases may explain why rats that inhale particulate materials that cause inflammation, but do not act directly on DNA, have a high incidence of lung cancer.

Mutations in p53 can also reveal that an individual has an increased susceptibility to cancer owing to inheritance of a germline mutation, a concept first proposed for the retinoblastoma (Rb) tumor suppressor gene [32]. Germline p53 mutations are missense and occur frequently in the cancer-prone individuals with Li-Fraumeni syndrome [33]. Laboratory animals with either a mutant p53 transgene or a deleted p53 gene, that is, homozygous or heterozygous 'gene knockouts', also are particularly susceptible to cancer [34,35]. These mutations in p53 are associated with instability in the rest of the genome [36]. Such instability could generate multiple genetic alterations leading to cancer. Indeed, genomic instability (including gene amplification) increases in frequency in cells that lack a normal p53 gene [37,38]. Furthermore, loss of the wild-type alleles of the p53 gene abrogates DNA damage-induced delay of the cell cycle in G1 [39]. DNA repair of certain promutagenic lesions can proceed prior to DNA synthesis in S phase. Less time for repair would increase the frequency of mutations. Since p53 is an integral component in one pathway of programmed cell death (apoptosis) induced by DNA-damaging chemotherapeutic drugs or ionizing radiation [40,41], inactivation of p53 could increase both the pool of proliferating cells and the probability of their neoplastic transformation by inhibition of programmed cell death.

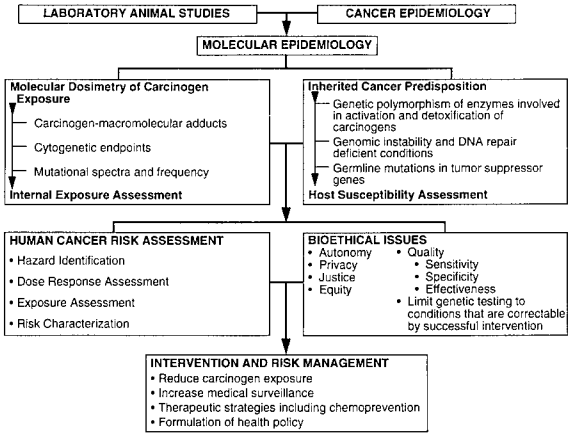


Fig. 3. Human cancer risk assessment and bioethical issues associated with molecular epidemiology and human cancer.

Such progress in the fields of molecular carcinogenesis and molecular epidemiology increases our ability to accurately assess cancer risk (Fig. 3). Cancer risk assessment, a highly visible discipline in public health, has historically relied on classical epidemiology, from chronic exposure of rodents to potential carcinogens, and the mathematical modeling of these findings. The field has been forced to steer a prudent course of conservative risk assessment because of limited knowledge of the complex pathobiological processes during carcinogenesis: differences in the metabolism of carcinogens, different DNA repair capacities, variable genomic stability among animal species, and variation among individuals with inherited cancer predisposition have made definitive analysis of cancer risk almost impossible [5,42]. Because regulatory decisions based on cancer risk assessments have significant public health and economic consequences, the scientific basis of risk assessment continues to be, and should continue to be, actively investigated [43].

Many questions remain. Are the pathways of

molecular carcinogenesis similar in rodents and humans? Because the time to develop cancer is generally shorter in rodents than in humans, could the apparent interspecies differences be due to the number of genetic and epigenetic events required for malignant progression or to the rate of transit between the events? Is the more frequent mutation of the *ras* proto-oncogenes in rodent, as compared to human, cancer a reflection of a pathway that is parallel and equivalent to the p53 pathway in human carcinogenesis? Are the selective pressures for clonal expansion of preneoplastic and neoplastic cells in human carcinogenesis similar to those in animal models?

Investigations of the p53 tumor suppressor gene are an example of the recent progress in molecular aspects of cancer research. A better understanding of molecular carcinogenesis and molecular epidemiology will eventually decrease the qualitative and quantitative uncertainties associated with the current state of cancer risk assessment and improve public health decisions concerning cancer hazards. Indeed, determination of the type and number of mutations in p53 and other cancer-related genes in tissues from 'healthy' people may allow the identification of those at increased cancer risk and their consequent protection by preventive measures.

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Cell proliferation as a determining factor for the carcinogenicity of chemicals: studies with mutagenic carcinogens and mutagenic noncarcinogens

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Abstract

Recent work in our laboratory has examined mechanisms whereby chemicals produce mutagenicity in short-term in vitro assays yet fail to produce carcinogenesis in 2-year rodent bioassays. These studies have used mutagenic structural analogs of carcinogenic and noncarcinogenic chemicals for comparison. Our previous studies have determined that differences in the metabolism and disposition of these chemicals were not responsible for their observed carcinogenic differences, but that carcinogenicity correlated with the ability of the respective isomer to induce cell proliferation in the target organ. Mutagenic noncarcinogens such as 2,6-diaminotoluene (DAT), 1-nitropropane (NP), dimethoate, dioxathion, and dichlorvos failed to induce an increase in cell turnover in the target organs. An increase in cell proliferation was observed following exposure to the mutagenic carcinogen analogs 2,4-DAT (liver), 2-NP (liver), and *tris*(2,3-dibromopropyl)phosphate (kidney). Our recent studies have used transgenic (Big Blue®) mice to detect in vivo mutagenesis induced by DAT isomers. Results of these studies demonstrate that administration of the carcinogenic isomer, 2,4-DAT, resulted in an increase in in vivo mutation frequency, whereas administration of the noncarcinogenic isomer, 2,6-DAT, failed to do so. These results indicate that cell proliferation may be requisite for expression of chemical-induced mutagenicity in vivo and thereby accommodate expression of carcinogenicity.

Keywords: Cell proliferation; Mutagenic noncarcinogens; Mutagenic carcinogens; Diaminotoluenes; Nitropropanes; Organophosphates; Big Blue® transgenic mouse system

In an effort to predict the carcinogenicity of chemicals more quickly and cheaply than can be achieved using a 2-year rodent bioassay, numerous short-term assays have been developed. The most commonly used assay is the *Salmonella* mutagenicity test which consists of a bacterial test strain, the chemical of interest, and a metabolic activation system [1]. Recent analysis has demonstrated that the data from this test were in concordance with the results from 2-year rodent bioassays as often or more often than were data from other commonly used short-term assays [2,3]. However, the concordance between the *Salmonella* mutagenicity test and rodent bioassays for carcinogenesis is still much less than desirable. Two major

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groups of chemicals were identified where short-term test data did not agree with data from rodent bioassays: (1) chemicals which were not mutagenic in the *Salmonella* test yet were found to induce cancer in rodents; and (2) chemicals which were positive mutagens in the *Salmonella* test yet failed to induce cancer in rodents. The first group was termed 'false negatives' and the second group 'false positives' due to the discordance between results in short-term tests and rodent bioassays. This discordance between results from in vitro short-term tests and in vivo bioassays decreases the confidence and therefore the value of short-term testing for the prediction of carcinogenicity. Our approach to understanding the discordance between such in vitro and in vivo test results has been to study pairs of structurally related compounds, both of which are mutagenic in short-term assays, yet one is carcinogenic and one noncarcinogenic in NTP bioassays. We sought differences in the way rodents respond to treatment of these pairs of chemicals in order to gain insight into the processes resulting in the carcinogenicity of one of the pair and the lack of carcinogenicity of the other.

Research in our laboratory demonstrated that one difference between the mutagenic carcinogens and the mutagenic noncarcinogens was in the ability of the carcinogens, but not the noncarcinogens to induce cell proliferation in the target organ for carcinogenesis [4–7]. These results provide support for the hypothesis that chemically induced cell proliferation is associated with carcinogenesis. Similar conclusions have been made by other investigators [8,9].

It is generally accepted that carcinogenesis is a multi-step phenomenon, including the stages of initiation, promotion, and progression. The carcinogen and the noncarcinogen in the pairs we studied must therefore produce opposite effects in the rodent at one of these steps in order to explain the reason for their opposite effects in the NTP bioassay. Our initial studies tested the mutagenic hepatocarcinogen 2,4-diaminotoluene (2,4-DAT) and the mutagenic noncarcinogen 2,6-diaminotoluene (2,6-DAT) for differences in their disposition and metabolism (Fig. 1). We

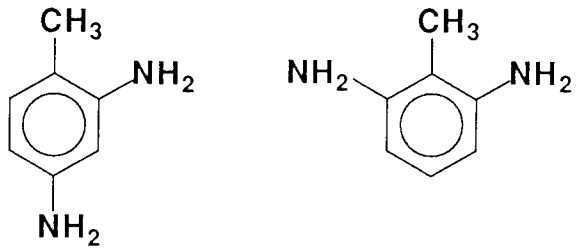


Fig. 1. Structures of 2,4- and 2,6-diaminotoluene.

reasoned that a possible explanation for the observed difference in their carcinogenicity may result from poor absorption or low metabolic activation of the noncarcinogenic 2,6-DAT as compared to the carcinogenic 2,4-DAT. Results of these studies demonstrated no differences in the rate of absorption or the degree of metabolism between these 2 compounds [10].

The next step in the multi-step process of carcinogenesis is a round of cell replication which fixes the genetic lesion in at least one of the daughter cells and prevents DNA repair enzymes from repairing errors. Using immunohistochemical techniques, we demonstrated that the carcinogenic isomer 2,4-DAT produced a dose-related increase in cell proliferation in rat liver, whereas the noncarcinogen 2,6-DAT produced no increase in cell proliferation in the liver, even at twice the dose of 2,4-DAT. The doses selected for our studies were equivalent to the doses used in the NTP bioassay [4].

We also observed similar results with the mutagenic hepatocarcinogen 2-nitropropane (2-NP; Fig. 2) and the mutagenic noncarcinogen 1-nitropropane (1-NP; Fig. 2). 2-NP is mutagenic in in vitro tests and produced benign and malignant liver tumors in male Sprague–Dawley rats after 26-week gavage exposure [11]. We demonstrated that 2-NP produced significant, dose-related cell proliferation in the liver in rats. The mutagenic structural analog, 1-NP, did not produce hepatic tumors after a 26-week gavage study similar to that for 2-NP, and was shown to



Fig. 2. Structures of 1- and 2-nitropropane.

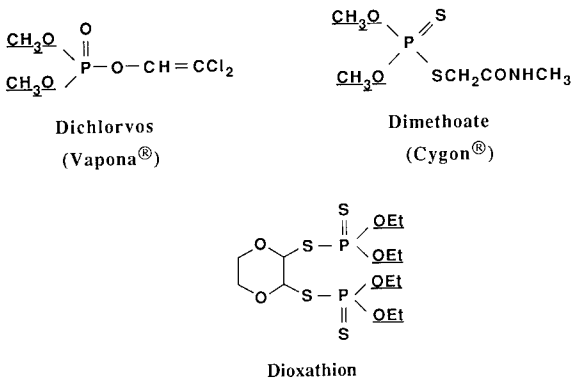


Fig. 3. Structures of mutagenic noncarcinogens dichlorvos, dimethoate and dioxathion.

produce no increase in hepatic cell proliferation above control [5].

Dichlorvos, dimethoate, and dioxathion (Fig. 3) were chosen for study because they were positive in short-term in vitro bacterial mutagenesis assays and possess structural alerts for carcinogenicity [12], yet were not carcinogenic in the liver or kidney in 2-year bioassays [7]. They are widely used organophosphate insecticides for application on agricultural crops and livestock. Human exposure may occur with agricultural workers as well as those involved in their manufacture.

These are structurally similar to the organophosphate flame retardant *tris*(2,3-dibromopropyl)phosphate (TRIS) which is also mutagenic, possesses structural alerts for carcinogenicity as determined by Ashby and Tennant [12] (Fig. 4), and was shown to be a potent renal carcinogen in long-term feeding studies [6].

The observation that TRIS induces cell proliferation only in the outer medulla of the kidney prompted a further histopathological review of the kidneys of the TRIS-exposed animals. Results of this further evaluation indicated the renal adenomas and adenocarcinomas were found almost exclusively in the outer medulla following 2-year feeding studies and provided strong evidence for the role of cell proliferation in the renal carcinogenesis induced by TRIS [6]. The observation that cell proliferation may be tissue or cell type-specific may, therefore, be of predictive value. Information on the mutagenicity of a chemical, in addition to data on the site of induction of cell proliferation, may prove useful for the prediction of carcinogenicity and its localization within a tissue or organ. The observation that the dose-response relationship for cell proliferation in this study and the incidence of renal tubular carcinogenesis were both flat is very interesting. This suggests that a maximal effect occurred in both the carcinogenesis and cell proliferation studies at 50 ppm which may have resulted from saturated GI absorption or renal metabolism of TRIS.

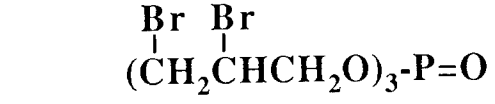


Fig. 4. Structure of the mutagenic renal carcinogen TRIS.

Transient bursts of cell proliferation, up to several weeks, may be sufficient to produce the conditions favorable for carcinogenesis. This may be especially true for mutagenic chemicals. The combination of mutagenicity and at least a transient round of cell replication may enhance the induction of carcinogenesis. In support of this concept, it was observed that the first identifiable step in diethylnitrosamine hepatocarcinogenesis is the appearance of altered foci containing glutathione transferase-placental form ($GST\text{-}P^{+}$) which was demonstrated to occur in the same hepatocytes which stain positive for BrdU [13]. These data suggest that induced cell proliferation may act in the initiation stage of chemical carcinogenesis, at least for mutagenic chemicals. Nonmutagenic carcinogens, such as Wy-14643, mirex, perfluorooctanoic acid or methapyrilene may require more sustained levels of cell proliferation in order to induce carcinogenesis than are required for mutagenic chemicals [14–17]. Clearly, more research is needed to evaluate the relevance of transient versus sustained cell proliferation in the carcinogenicity of mutagenic and nonmutagenic chemicals.

Several lines of evidence suggest a mechanistic basis for the relationship between enhanced cell replication and chemical carcinogenesis: (1) enhanced cell replication increases the number of

cells in S phase which is a sensitive time in the cell cycle during which DNA is more vulnerable to the damaging effects of chemicals, and limits the time for DNA repair before mutations become permanent [18]; (2) enhanced cell replication per se may also result in oncogene activation produced by hypomethylation of chromatin [19] and (3) increased cell proliferation may also promote the clonal expansion of preneoplastic cells during the promotion and progression stages in multi-stage carcinogenesis models [8,20].

Our recent studies with 2,4- and 2,6-DAT using newly developed transgenic mouse lines have provided further insight into the relationship of cell proliferation and chemically induced mutagenesis [21]. Transgenic mouse mutagenesis assays represent a novel approach for assessing the mutagenicity of various compounds. Before the availability of these assays, the mutagenic properties of a chemical were often determined using only short-term in vitro tests. The Big Blue® assay represents an opportunity to examine the in vivo mutagenic properties of deleterious agents through the use of a stable genomic integration of the lambda (λ) shuttle vector (λLIZ) which carries a *lacI* target gene and a *lacZ* reporter gene [22,23]. After treatment of mice with the agent in question, genomic DNA is isolated from the mouse target organ(s) and the λ shuttle vector is recovered using in vitro phage packaging extracts. Infection of *E. coli* SCS-8 cells followed by expression of the *lacI* and *lacZ* genes permits the detection of phage-carrying mutated *lacI* genes. If the normal function of the *lacI* repressor is disrupted, the *lacZ* gene product, β-galactosidase, is expressed resulting in the generation of blue plaques on plates containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) [22]. Scoring the ratio of these blue mutant plaques to colorless, nonmutant plaque allows for the quantitative measure of mutant frequency (MF) in the tissue of interest. We examined the *lacI* mutant frequencies induced after exposing male B6C3F1 mice to 2,4- and 2,6-DAT in feed. We demonstrated a 2-fold increase in MF at the 90-day time point in the 2,4-DAT-treated animals that was significant ($P < 0.01$) as compared to either the untreated controls or the 2,6-DAT-treated animals. That 2,4-DAT but not 2,6-DAT was found to be mutagenic in vivo is interesting in light of previous studies conducted in this laboratory in which we demonstrated that 2,4-DAT, but not 2,6-DAT induces hepatocellular proliferation [4]. We postulate that a cytoproliferative effect in target organs could produce an elevated MF in Big Blue® mice as a result of clonal expansion of chemically mutated cells. Cells carrying DNA damage from either 2,4- or 2,6-DAT treatment would yield mutations if forced to replicate. However, hepatocellular proliferation is only induced by 2,4-DAT. The cytotoxicity and compensatory cell replication induced by 2,4-DAT treatment may be required for mutation fixation to occur. Such damage effectively increases the mutation rate (mutants/cell/generation) leading to the elevated MF (mutants/viable phage) observed in 2,4-DAT-exposed mice at 90 days. Such a mechanism could also be involved in the promotion and progression stages of carcinogenesis [20]. The noncarcinogenic isomer 2,6-DAT did not increase the MF in either the 30- or 90-day animals at a treatment dose identical to the 2,4-DAT treatment dose. Thus, 2,6-DAT may not cause an increase in DNA damage in mouse liver and may only show a mutagenic response in vitro. However, since the proliferative effect has not been observed in 2,6-DAT-exposed animals [4], it is also conceivable that DNA damage may have been induced in the liver by 2,6-DAT exposure but that such damage was repaired in the absence of cellular replication, with a corresponding reduction of the MF to background levels.

Data presented in this overview indicate a positive association between increased cell proliferation and hepatocarcinogenesis, and point out the value of performing mechanistic studies such as cell proliferation assays in conjunction with short-term tests for explanation of the results of NTP bioassays. Mechanistic studies such as cell proliferation assays may also aid in the selection of doses for future bioassays and in the process of human risk assessment in the use of the NTP

bioassay data. Though some nonmutagenic chemicals induce increased tumor incidences and are also associated with increased cell proliferation, we do not intend to infer that cell proliferation alone is sufficient to induce an increased incidence of tumors. Numerous chemicals have been shown to induce sustained cell proliferation in the absence of an increased tumor incidence [24]. Based on the data from our laboratory, it is our hypothesis that whereas cell proliferation may not be sufficient to induce carcinogenesis, it creates a favorable environment for tumor development. It is this favorable environment which permits the manifestations of the mutagenic activity of any chemical that may be present either in the form of the chemical administered, one or more of its metabolites or as other naturally occurring or foreign chemical(s). It is clear from data presented here that mechanistic studies to understand the relationship of sex, species and dose in rodent carcinogenicity assays of chemicals is critical for the extrapolation of such data for human health assessments [25].

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Toxicology Letters

Role of cell proliferation in regenerative and neoplastic disease

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Abstract

DNA replication does not have 100% fidelity. Consequently, a chemical can increase the risk of cancer either by directly damaging DNA (genotoxic) or by increasing the number of cell replications, or both. Increased cell proliferation can be produced by increasing cell births (by direct mitogenesis or regeneration following toxicity), or decreasing cell deaths (by inhibiting apoptosis or differentiation). Cell proliferation can affect the dose-response curve for genotoxic carcinogens and is the basis for carcinogenicity by nongenotoxic agents. Bladder carcinogens will be used to illustrate these mechanisms, and their implications with respect to human risk assessment will be presented.

Keywords: Cell proliferation; Bladder carcinogenesis; Sodium saccharin; Acetylaminofluorene; Calculi

1. Introduction

Carcinogenesis proceeds through a sequence of genetic alterations [1–3]. In addition, there are multiple genetic and nongenetic mechanisms for altering susceptibility to carcinogenesis. Furthermore, DNA replication does not have 100% fidelity, although, fortunately for our survival, the rate of mistakes per cell division is extremely small. Although the error rate is not precisely known, it has been estimated at approximately one mistake per 10^{10} nucleotides per cell division. It is also critical that the genetic alterations occur in the pluripotential (stem) cell population of a target tissue. Genetic alterations to cells that are already in the process of differentiation are essentially committed along a pathway to cell death.

There are only two basic means by which an agent can alter the rate of carcinogenesis in a given cell population [1–3]. To increase the risk of cancer, an agent can either increase the rate at which mistakes occur in DNA replication during each cell division or an agent can increase the number of cell divisions in the target pluripotential cell population. Of course, an agent can do both. Agents which alter the rate of DNA damage per cell division are genotoxic, and most such agents at sufficiently high doses also increase the number of cell divisions. Agents which only increase the number of cell divisions without affecting the rate of DNA damage per cell division are considered nongenotoxic.

Numerous pathways for the metabolic activation of chemicals have been identified which result in the production of DNA adducts [4]. These pathways include a variety of enzymatic functions of the various P450 isozymes, but can also involve a variety of other enzymes including prostaglandin H synthase, various peroxidases and oxidases, glutathione transferases, and many others. Some DNA adducts can produce muta-

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tions. If the mutation happens to occur in a gene required for the development of cancer, the risk of cancer is increased. DNA adducts also can be repaired [5]; however, the risk of cancer is only increased if mutation occurs.

Some of the reasons for the lack of 100% fidelity during DNA replication have begun to be identified (Table 1). Some of these result from formation of endogenous DNA adducts, most of which are repaired; some do not produce a very high rate of mutation. It is only if the lesion is not repaired and leads to mutation in a gene required for the development of cancer that the risk is increased.

Whether the defect is caused by an endogenous process or due to exogenous exposure producing DNA damage, DNA replication is required to fix the genetic mistake permanently [1–3]. Thus, DNA replication not only provides more opportunities for mistakes to happen, but fixes those that do occur into a permanent alteration in the genome. It is this increase in the number of DNA replications that leads to an increased risk of cancer over the lifetime of the organism.

It is essential to note that it is an increase in the number of DNA replications that is of critical importance, not necessarily the rate at which they happen [1–3,6]. The formula for the labeling index is as follows:

$$\text{Labeling index} = \frac{\text{Number of cells undergoing DNA replication}}{\text{Total number of cells}} \quad (1)$$

Since the key component is number of replications, it can be rewritten as follows:

Table 1
Endogenous factors

Replication errors (mismatch repair)
Oxidative damage
Depurination (depyrimidination)
Deamination
Inappropriate alkylation
Nitric oxide
Exocyclic adducts
Gratuitous DNA turnover

$$\text{Total number of DNA replications} = \text{Labeling index} \times \text{Total number of cells} \quad (2)$$

Thus, the total number of cell divisions can be increased by either increasing the labeling index or increasing the total number of cells. Most commonly, both of these parameters are increased, as in hyperplasia, multiplying the effect.

Occasionally an apparent discrepancy in the relationship of cell proliferation and carcinogenesis is found, but can be readily explained in terms of this formula. For example, monuron produces severe hepatocellular and generalized toxicity in mice, with an apparent increase in the proliferation rate of the liver, but is not carcinogenic [2,3]. The toxicity is so severe that the total size of the liver is approximately one-half that of the controls. Since the total number of hepatocytes is approximately one-half, the labeling index would need to be increased twofold just to produce a total number of cell divisions equal to the control level.

Administration of the peroxisome proliferator, diethylhexylphthalate (DEHP) produces only a transient increase in labeling index in the liver, and yet is a weak hepatocellular carcinogen [2,3]. It has been inferred that a persistent increase in cell proliferation has not occurred. However, the total number of hepatocytes increases following DEHP administration; thus, the total number of cell divisions is increased. Furthermore, DEHP appears to have a selective proliferative advantage for cells which have already undergone one of the genetic alterations leading to cancer, and this has a greater effect than simply affecting normal hepatocytes.

Just as there are numerous pathways to produce DNA adducts and ultimately mutations, there are multiple pathways by which the number of DNA replications can be increased [2,6]. Essentially, they can be classified into two general groups of effects: those which increase the number of cell births and those that decrease the number of cell deaths. An increase in cell births can be produced by either a direct mitogenic effect or by cell toxicity with regenerative hy-

perplasia. Direct mitogenicity usually involves a hormonal or growth factor interaction with a specific cell receptor and usually requires low doses. Cytotoxicity can be produced by a variety of mechanisms, including damage to cell membranes, cytoplasmic or nuclear components, as well as DNA. If an adequate amount of chemical is not administered to produce the toxicity, there is no increase in cell proliferation.

A decrease in cell deaths can be produced by either inhibiting apoptosis and/or inhibiting cell differentiation [2,6]. Again, the ultimate effect must occur in the stem cell population, and if there is a blockage in the percentage of cells that either die or undergo differentiation (which is also a death process), the number of cells in the stem cell population increases. Again, referring to formula (2) above, although the labeling index might not increase, the number of cells in the stem cell population increases and, therefore, there is a net increase in the number of replications.

2. Genotoxic carcinogens – 2-acetylaminofluorene (AAF)

In the late 1970s, an experiment was performed at the National Center for Toxicological Research utilizing a large number of mice to detect a statistically significant increase in tumor incidence of 1% [7]. AAF was administered in the diet at doses ranging from 30 ppm to 150 ppm extending for 33 months, inducing tumors of the liver and bladder. The incidence of liver tumors had an apparent linearity, whereas the bladder had an apparent no-effect level below 60 ppm. This provided an enigma regarding interpretation of low-dose extrapolation for genotoxic carcinogens, which was compounded when Beland and associates [8] demonstrated that the steady state DNA adduct levels for AAF in liver and bladder were linear to doses as low as 5 ppm, with the number of adducts actually greater per unit of DNA in the bladder than in the liver. One interpretation is that the DNA adducts are irrelevant to carcinogenesis. This is highly unlikely since there is considerable research demonstrating that DNA adducts are germane to the carcinogenic process by inducing mutations.

In the mouse liver, hepatocytes replicate at a relatively slow rate, and the doses used in the ED_{01}-study did not increase either their rate of proliferation or number [9]. However, AAF in the liver is metabolized by N-hydroxylation followed by sulfation, which leads to a reactive electrophile and the formation of mutagenic, C-8 guanine adducts. N-hydroxylation occurs in normal hepatocytes but not in hepatocytes which have already undergone at least one of the steps toward the carcinogenic process. Thus, the rate of DNA damage is affected only in normal cells, and cell proliferation indices remain at normal levels. It is not too surprising that tumor formation is approximately linear.

Following N-hydroxylation, in addition to sulfation, the liver can also N-glucuronidate the amine, which is excreted in the urine and hydrolyzed to the free hydroxylamine. The latter can interact with the DNA of any cell, whether normal, intermediate or tumor urothelial cells. Similar to the normal mouse liver, the normal mouse bladder epithelium undergoes replication at a low rate, turning over approximately every 100–200 days. In addition, there are considerably fewer bladder epithelial cells than there are hepatocytes. At doses below 60 ppm, AAF does not increase the number or rate of proliferation in these bladder cells. However, at 60 ppm and above, hyperplasia is induced, which represents an increase in the number of bladder epithelial cells and an increased rate of cell division, resulting in a marked increase in the number of DNA replications. This increased number of DNA replications provides an increased number of targets for the AAF reactive metabolites as well as an increased number of cycles in which the mutations can be fixed irreversibly. At doses of 60 ppm and above there is a detectable increase in the incidence of bladder tumors in mice treated with AAF. At doses below 60 ppm, there is likely to be an increase in bladder tumor incidence since there are DNA adducts formed, but the increased incidence is less than the 1% detection limit of the experiment. The carcinogenicity of AAF in the bladder is not depen-

dent on an increased cell proliferation rate, but the dose-response for tumor incidence is greatly affected by the rate of replication. A similar effect is seen in the liver, but only at higher doses than those used in the ED_{01}-experiment.

3. Nongenotoxic bladder carcinogens

For chemicals such as AAF, DNA adduct formation can be detected at considerably lower doses than an increased tumor incidence, suggesting that there is a potential carcinogenic effect even at very low doses. This has given rise to the concept of a no-threshold phenomenon for carcinogens. This is a possibility for genotoxic carcinogens [1–3].

In contrast, nongenotoxic carcinogens, especially those not acting through a specific cell receptor, can have a true biologic threshold [1–3]. Many forms of cell toxicity are particularly well known that involve threshold phenomena. Since the toxicity frequently is *required* for induction of regenerative hyperplasia, the carcinogenic process also must be a threshold phenomenon.

The most frequent cause of toxicity and consequent regenerative hyperplasia and carcinogenesis in the bladder is formation of urinary calculi [2,3,11]. Numerous chemicals have been identified that can produce urinary tract calculi, and many of these are urinary tract carcinogens in rodents (Table 2). The rate of tumor formation appears to be related to the coarseness of the calculus or implanted pellet and to the extent of trauma to the urothelium and consequent regenerative hyperplasia [10].

If adequate amounts of the administered chemical and/or a metabolite result in the formation of calculi, regenerative hyperplasia and tumors occur [2,3]. Many of the substances (Table 2) that produce urinary tract calculi are actually essential for cellular and organismal viability, such as calcium, uracil, and others. Alterations of normal physiology can also produce calculi, as occurs with various forms of hyperoxalosis or surgical portacaval shunt formation in rats. The latter produces an alteration in uric acid metabolism and ultimately urinary tract calculi and tumors. Of greatest significance is the fact that a critical amount of the chemical is needed for the precipitation to occur in the urine. The amount needed can be greatly modified by other urinary components so that there is frequently a supersaturated solution present before there is actually precipitate formation. If the dose of the administered chemical does not produce a urinary concentration critical for the production of a calculus, no calculus will form, there is no toxicity, no regenerative hyperplasia, and no tumors. Obviously, this is a true threshold phenomenon based on chemical and physiological principles.

There is some evidence that the presence of urinary tract calculi in humans also increases the incidence of bladder tumors, particularly squamous cell carcinomas [12]. However, the rate is low since calculi in the human urinary tract frequently are either spontaneously voided or cause obstruction, resulting in excruciating pain and

Table 2
Chemicals administered at high doses in the diet which lead to urinary calculi in rodents

Uracil	Acetazolamide
Melamine	Terephthalic acid
Fosetyl-al	Dimethyl terephthalate
Biphenyl	Nitrilotriacetate
Glycine	Polyoxyethylene β-stearate
Orotic acid	Diethylene glycol
Oxamide	Calcium oxalate
Calcium phosphate	Uric acid
Homocysteine	4-Ethylsulfonylnaphthalene-1-sulfonamide

rapid removal by an urologist. Nevertheless, quite large quantities of most chemicals are required before there is calculus formation. If human exposure is well below the amounts needed for formation of the calculus, there is no human carcinogenic risk [2,3,11].

A subtler form of a similar phenomenon results from the administration of sodium saccharin or related salts to rats [13,14]. This appears to be not only a high dose phenomenon, but also is species specific. In the rat, there are a combination of critical factors generated in the urine when administered high doses of sodium salts which produces an amorphous urinary precipitate which is cytotoxic to urothelial cells and is composed predominantly of calcium and phosphate. The critical factors include high urinary concentrations of calcium and phosphate as well as a pH above approximately 6.5, high concentrations of urinary protein, and possibly high concentrations of mucopolysaccharides and other substances in the urine which may contribute to the formation of the precipitate. In the rat, there are extremely high levels of protein, with the concentration approximately twice as high in males compared to females because of the presence of α_{2u}-globulin after the age of approximately 6 weeks. The differences in urinary protein between males and females can readily explain the differences in both the proliferative and tumorigenic response to sodium saccharin. The importance of urinary protein can be seen by the decreased responsiveness to sodium saccharin and sodium ascorbate in NBR rats [15], which are without the high concentrations of α_{2u}-globulin in male rat urine, and in analbuminemic rats, which are without the high concentrations of albumin in male or female rat urine [16]. Also present are high concentrations of urinary calcium and phosphate, which increase following administration of high doses of sodium saccharin or other sodium salts, despite an overall dilution of the urine [17].

Mice have similar levels and types of protein as do rats, and yet do not respond with proliferation or tumors following administration of high doses of sodium saccharin [13,17]. This appears to be due to the fact that the urinary calcium and phosphate levels in mice are approximately 10- and twofold lower than in rats, respectively, and the concentrations decrease along with the overall dilution of the urine following consumption of high levels of sodium salts [17].

The importance of pH can be demonstrated by treatments that produce acidification of the urine, such as coadministration with ammonium chloride or administration of the sodium salt in AIN-76A diet [13]. Either treatment produces a urinary pH below six and inhibition of the proliferative or tumorigenic effects of sodium salts.

Similar results are seen with high doses of nearly any sodium salt [13,14]. As can be seen in Table 3, except for saccharin, all are natural compounds, many of which are essential for survival and most of which are formed endogenously as well as being available exogenously in the diet. Of critical importance is the need for extremely high doses of the salts even in the rat, with a no-effect level in the male rat of approximately 1% of the diet. Most importantly is the apparent species specificity of this effect; the only species that responds is the rat. Not only is the mouse refractive to urothelial effects following administration of high doses of sodium salts, but no urothelial effect has been seen in other species, such as hamster, guinea pig, and monkey. Recently, monkeys treated with sodium saccharin for greater than 20 years have been sacrificed, and were found to have no proliferative or tumorigenic effects in any tissue, including the urothelium [18]. Monkeys also do not form the amorphous urinary precipitate, pre-

Table 3
Sodium salts which produce urothelial hyperplasia and increase bladder carcinogenesis when fed at high doses to male rats

Saccharin	Ascorbate
Glutamate	Aspartate
Citrate	Erythorbate
Succinate	Phytate
Phosphate	Bicarbonate
Chloride	

sumably because of the low levels of protein in the urine [1–3,13].

In humans there is no evidence of the formation of amorphous urinary precipitate, and epidemiologic evidence shows no-effect in the urinary tract of humans, either proliferative or tumorigenic [13].

4. Interaction of genotoxicity and cell proliferation

For a genotoxic chemical, such as AAF, if administered at sufficiently high doses, there is both a genotoxic effect as well as a proliferative effect [1–3]. This frequently leads to a synergistic increase in tumorigenicity. This has been observed for a wide variety of chemicals in several target organs, such as diethylnitrosamine in liver and formaldehyde in nasal passages. Of critical importance in humans are simultaneous exposures to a variety of chemicals at various levels with different effects. A beginning has been made in evaluating such interactions.

In studies on urinary bladder cancer, administration of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT), a classic, genotoxic nitrofuran, produces a 100% incidence of tumors at high doses, but produces no detectable tumor incidence at doses below 0.01% of the diet [19]. Nevertheless, at lower doses, there presumably are DNA adducts formed although there is not an increased proliferative effect as seen at higher doses. If a very low dose of FANFT (0.005%) is administered simultaneously with another agent which increases cell proliferation, such as high doses of sodium saccharin (5% of the diet), a significant incidence of bladder tumors are produced. Thus, an interaction between genotoxicity and increased cell proliferation, whether produced by high doses of a genotoxic chemical or by administration of low doses of a genotoxic chemical with a nongenotoxic chemical, can lead to a detectable incidence of bladder tumors.

There is also a suggestion of a similar response in humans when multiple chemicals with different effects are administered. For example, cigarette smoke contains a small quantity (nanograms) of aromatic amines, such as 4-aminobiphenyl, a known human bladder carcinogen [20]. Based on previous exposure records in humans as well as animal experiments, this is an inadequate dose for producing a detectable incidence of bladder tumors, although cigarette smoking is the major etiologic factor in bladder tumor formation in humans in the United States, especially in males. Nevertheless, cigarette smoke also contains other chemicals, which have not yet been identified, which increase the proliferation of the bladder epithelium. The combination of genotoxicity by low doses of aromatic amines combined with the cell proliferative effects resulting from other agents in cigarette smoke can readily lead to the formation of a detectable incidence of bladder tumors, similar to the combination of FANFT and sodium saccharin in the rat.

5. Implications for risk assessment

Based on the above discussion and examples, chemicals can broadly be divided into genotoxic and nongenotoxic agents [1–3]. Nongenotoxic agents can be further divided into those that react through specific cell receptors and those that act through nonspecific means. For genotoxic chemicals, although the dose response may extend down to very low levels, and may actually involve a nonthreshold phenomenon, the dose-response for tumor incidence can be greatly affected by cell proliferative phenomenon as demonstrated for the bladder in the ED_{01}-study in mice. For nongenotoxic chemicals, particularly those acting by nonreceptor mechanisms, there will usually (if not always) be a threshold phenomenon involved, frequently associated with toxicity. This does not mean that all toxicities will produce tumors, as there are numerous types of toxicity that do not affect cell proliferation, especially in stem cell populations. It is only if there is a significant increase in the cell proliferation of stem cells that an increased tumorigenic response is likely. If the toxicity involves a threshold phenomenon then the tumorigenicity, dependent on the increased cell proliferation of regenerative proliferation, also involves a threshold phenomenon. For nongenotoxic chemicals,

especially if a threshold mechanism can be identified, such as for calculus-forming chemicals or sodium salts, a different type of risk assessment is required than for genotoxic chemicals when extrapolating to low dose human exposures. This has been incorporated into the guidelines of the United States Environmental Protection Agency in evaluating carcinogenic hazard to humans of various chemicals based on rodent bioassays. Although the rodent bioassay [3] can be of great importance in identifying potential human carcinogens as well as increasing our understanding of the carcinogenic process, it must be understood that the long-term rodent bioassay is merely a screening assay, and that many chemicals identified as carcinogens in rodents when administered in high doses will not be carcinogenic to humans at low dose exposures, especially with respect to nongenotoxic chemicals. Mechanistic information that is available must be incorporated into the assessment.

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The role of regenerative cell proliferation in chloroform-induced cancer

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Abstract

Chloroform produces cancer by a nongenotoxic-cytotoxic mode of action, with no increased cancer risk expected at noncytotoxic doses. The default risk assessment for inhaled chloroform relies on liver tumor incidence from a gavage study with female $B6C3F_1$ mice and estimates a virtually safe dose (VSD) at an airborne concentration of 0.000008 ppm of chloroform. In contrast, a 1000-fold safety factor applied to the NOAEL for liver cytotoxicity from inhalation studies yields a VSD of 0.01 ppm. This estimate relies on inhalation data and is more consistent with the mode of action of chloroform.

Keywords: Chloroform; Nongenotoxic carcinogen; Cell proliferation; Risk assessment

Chloroform is a common environmental pollutant produced by some industrial processes and in trace amounts from the chlorination of drinking water. Inhalation and ingestion are the most frequent routes of exposure. Chloroform induces liver cancer in male and female $B6C3F_1$ mice when administered by gavage, kidney cancer in male, but not female, Osborne–Mendel rats when given by gavage or in the drinking water, and kidney cancer in male BDF_1 mice, but not female BDF_1 mice or either sex of F-344 rat when administered by inhalation [1–3].

Many carcinogens induce cancer by reacting with DNA to produce mutations in growth control genes [4]. Risk assessments for such genotoxic agents are very conservative and assume a linear response even at vanishingly small doses [5]. In contrast, chloroform appears to be acting through a nongenotoxic-cytotoxic mode of action [6].

Chloroform has been subjected to a number of genotoxicity assays, and a thoughtful evaluation of these data requires more than a superficial listing of the number of positive and negative tests. For example, 20 negative Ames mutagenicity studies with chloroform have been published in the literature. An expert committee convened by the International Programme on Chemical Safety (IPCS) of the World Health Organization recently published a critical review in which they concluded that neither chloroform nor its metabolites appear to interact directly with DNA or possess genotoxic activity [7]. A

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complication with evaluating genotoxicity studies is that the mutagenic effects of chloroform may be secondary to events associated with cytolethality, and interpretation of genotoxic activity in vivo must be done in light of the corresponding dose response for induced regenerative cell proliferation. The consistent negative response of chloroform in most genotoxicity assays including the sensitive Ames bacterial mutagenicity test, mutagenicity in *E. coli*, mutagenicity in Chinese hamster V79 cells, and chromosome breakage in human lymphocytes indicates a lack of direct covalent interaction between chloroform or its metabolites and DNA [7]. A sensitive target for chloroform-induced cancer is the female $B6C3F_1$ mouse liver [1]. Chloroform does not induce DNA repair in vitro or in vivo in hepatocytes from female $B6C3F_1$ mice, even at carcinogenic doses [8]. The oxidative metabolite of chloroform is phosgene, which has been demonstrated by chemical trapping. Phosgene is unlikely to react with any sites on DNA because of its rapid reactivity with water to produce carbon dioxide, hydrochloric acid and water [9]. Although no phosgene-DNA adduct is readily plausible under physiological conditions [9], any potential adduct that might form would be expected to be of a size and type that would induce a DNA repair response readily detected by these assays [10]. Chloroform also lacks initiating activity in initiation-promotion assays [7].

In those few cases of reported genotoxic activity, there are concerns with inconsistencies of the results with other findings or with idiosyncrasies of the particular system. For example, chloroform has been reported to be weakly mutagenic in the mouse lymphoma mutagenesis assay [11]. But results were inconsistent from one trial to the next, and weak activity was seen only at toxic concentrations where less than 10% of the cells survived. This assay commonly gives false positive results under such conditions [12]. There is one report of very weak binding of ^{14}C-labeled chloroform to DNA in every tissue examined including liver, kidneys, lungs, and stomach of male Wistar rats and male Balb/c mice exposed by intraperitoneal injection [13]. These data are not consistent with other DNA binding studies [14] and do not reflect the pattern of target organs for chloroform-induced tumors. For example, these binding data might suggest multiple target tissues in rats and mice. Yet a recent chloroform cancer study with male and female F-344 rats and male and female BDF_1 mice produced tumors only in the male BDF_1 mouse kidney, a site known to be very sensitive to the cytotoxic effects of chloroform [3]. It seems most likely that metabolic incorporation of one-carbon metabolites of chloroform is responsible for the small amount of radioactivity associated with DNA, rather than covalent adduct formation [13].

Critical examination of both the genotoxicity data and patterns of toxicity leads to the conclusion that chloroform produces cancer through a nongenotoxic-cytotoxic mode of action [4,7,15–20], that is, tumors are induced secondarily to events associated with toxicity and regenerative cell proliferation. Thus no increased risk of cancer would be expected below doses that do not produce toxicity [6].

The doses employed in cancer studies with chloroform have been criticized as being excessive and not relevant to common environmental exposures. In every case of chloroform induced-cancer examined thus far, there has been a corresponding association with a cytotoxic response [3,15,16,18,19]. Interestingly, the mouse liver tumor response disappears if the same daily gavage dose of chloroform is given in the drinking water instead. Cytotoxicity and regenerative cell proliferation are seen in the mouse liver when chloroform is given by gavage but not when given in the drinking water, suggesting that tumor formation is secondary to these effects [19]. Dosimetry studies suggest that toxicity and cancer are not observed when chloroform is given in the drinking water because the compound is not delivered to the liver or converted to toxic metabolites at a rate sufficient to kill hepatocytes.

Complete loss of carcinogenic activity by simply changing the vehicle and dose rate, as was seen in the gavage vs. drinking water studies, would not be expected of a mutagenic carcinogen. It must be remembered that the mice in

the drinking water study were exposed chronically to massive amounts of chloroform. Were chloroform directly mutagenic, irreversible mutations would accumulate even if they were formed at a slower rate, as is the case for mutagens and radiation. Lack of a carcinogenic response in the drinking water study is, perhaps, the strongest evidence of all that chloroform is not a directly genotoxic carcinogen.

An Osborne–Mendel male rat kidney tumor response was seen when chloroform was given in the drinking water, but only at the highest concentration of 1 800 000 ppb, which also resulted in a 60% decrease in water consumption and a 25% loss in body weight [2]. The nutritional status of this high-dose group was uncertain, and the validity of extrapolation of the tumor response to lower doses is questionable.

Chloroform administered to B6C3F$_1$ mice and F-344 rats by inhalation for up to 90 days produced concentration-dependent responses for toxicity and regenerative cell proliferation that are consistent with and predictive of the patterns of induced tumors in a recently completed 2-year chloroform inhalation study [3,21,22]. Chloroform also produces toxicity and cell proliferation in the nasal turbinates of rats and mice, but these lesions do not progress to cancer [3,22,23].

Integration of the above data with knowledge of dosimetry yields a cohesive explanation of the patterns of chloroform-induced cancer that is appropriate to each route of administration and consistent with a nongenotoxic-cytotoxic mode of action. Chloroform serves as an example for formulating appropriate risk assessments for carcinogens acting through a nongenotoxic-cytotoxic mode of action. Quantitative linking of dosimetry, cytolethality, regenerative cell proliferation, and tumor formation provides a framework for testing potential cause-and-effect relationships and for deriving appropriate risk assessments. The United States Environmental Protection Agency currently uses the linearized multistage model applied to the B6C3F$_1$ mouse liver tumor data from the chloroform gavage study to estimate a virtually safe dose (VSD) as an increased lifetime risk of cancer of one in a million. The resulting value is an airborne exposure concentration of 0.000008 ppm (0.04 $\mu g/m^3$) [24,25]. Assuming that chloroform-induced female mouse liver cancer is secondary to events associated with necrosis and regenerative cell proliferation, then no increases in liver cancer in female mice would be predicted at the no-observed-adverse-effect-level (NOAEL) of 10 ppm or below, based on the 90-day inhalation toxicity and cell proliferation study [21,22]. Applying an uncertainty factor of 1000 yields an estimate of a VSD at an airborne concentration of 0.01 ppm. This estimate relies on inhalation data and is more consistent with the mode of action of chloroform.

The IPCS expert committee endorsed this approach for chloroform risk assessments in cases where the critical supporting data were available, and used the same approach and safety factor as noted above to calculate the VSD or tolerable daily intake for orally administered chloroform [7]. The substantial difference in the VSD values for airborne chloroform of 0.000008 vs. 0.01 ppm relates to the use of conservative default assumptions applied in the absence of specific scientific information. The replacement of these default assumptions is consistent with the recommendations of the National Academy of Sciences/National Research Council committee report that revisited EPA's approach to assessing cancer risk of hazardous air pollutants [26]. In addition, the International Agency for Research on Cancer (IARC) also recently endorsed the use of mechanistic data to refine risk assessments and replace default assumptions for experimental carcinogens [27].

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Role of cell proliferation at early stages of hepatocarcinogenesis

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Abstract

Hepatocarcinogenesis in rodents is characterized by the early appearance of foci of enzyme-altered (initiated) cells which are believed to represent precursors on the way to malignancy. Proliferation of cells within enzyme-altered liver foci is generally increased as compared to surrounding normal hepatocytes. This may be mediated by changes in the rates of cell division and/or cell death (apoptosis). Cell proliferation is controlled by complex signaling networks and may be modulated by xenobiotics. In mouse – but not rat or human – liver, mutation of the Ha-*ras* gene appears to represent a critical genetic alteration which may confer a selective growth advantage to the mutated cells. Exogenous tumor-promoting agents may stimulate cell division and depress apoptosis of preneoplastic hepatocytes, thereby increasing the probability of cancer. By means of histochemical methods, data on the frequencies of both cell division and cell death can be collected separately and utilized for estimation of promoter efficacy. In addition, quantitative stereology may be applied to the analysis of the size distribution of enzyme-altered foci and used for modeling of hepatocarcinogenesis.

Keywords: Hepatocarcinogenesis; Cell proliferation; Apoptosis; *ras* mutations; Dioxins; TCDD

1. Introduction

The term cell proliferation is frequently used synonymously with cell division. Strictly speaking, however, this is not correct: proliferation denotes an increase in cell number, which may be mediated by changes in the rate of cell division, or in the rate of cell death, or both. During multi-stage carcinogenesis cell proliferation is one of the key determinators of the velocity of the process. Increases in cell division rates are associated with enhanced frequencies of spontaneous and carcinogen-induced mutations which, if critical cell regulatory genes are affected, increases the risk of cancer. At least as important are the kinetics of multiplication of 'initiated' tumor precursor cells at the intermediate stages of the carcinogenic process [1] (Fig. 1). Proteins inappropriately activated or inactivated as a consequence of mutation of their genes during initiation may permanently accelerate the proliferation of 'initiated' cells and their descendents by interfering with cellular control mechanisms of cycle time and time to natural death. Exogenous tumor-promoting agents may stimulate the proliferation of 'initiated' cells by mechanisms not principally different in nature. In our paper we will briefly describe the rodent liver system and deal with the effects of endogenous and exogenous factors capable of modifiying

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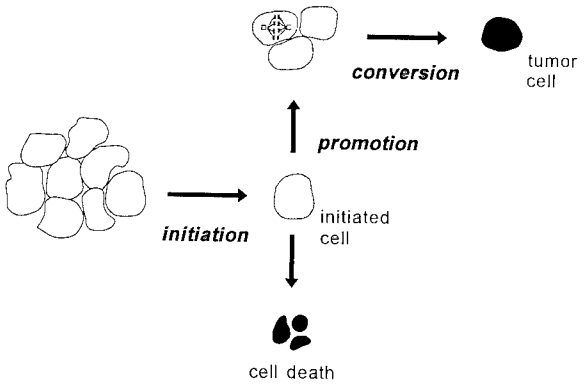


Fig. 1. Two-stage model of carcinogenesis (modified from [1]).

the proliferation of initiated cells in this system. Finally, some considerations regarding the categorization of carcinogens into 'initiators' and 'promoters' will be made.

2. Enzyme-altered foci (EAF) as early markers of hepatocarcinogenesis

The rodent liver offers an excellent experimental model to study the effects of xenobiotics on the proliferation of tumor precursor populations. Foci of hepatocytes characterized by alterations in the expression and activity of certain enzymes, such as adenosine triphosphatase, γ-glutamyltranspeptidase and glutathione *S*-transferase P (GST-P), occur shortly after treatment of rodents with carcinogens (for a review see [2]). There is accumulating evidence to suggest that at least some of the EAF represent precursor lesions which are causally related to the carcinogenic process in liver. Some important features of EAF are listed in Table 1.

The stereological analysis of EAF offers a valuable tool to analyze the kinetics of proliferation of enzyme-altered cells (see for example [3,4]. Since EAF are monoclonal in origin (see for example [5]), the number of foci gives a rough estimate of the initiating potency of a test agent. Promoter-mediated clonal expansion of cells within EAF leads to changes in the size distribution of these lesions in liver and may therefore serve as a rough estimate of their promoting activity. In addition to these (indirect) morphometrical procedures, biochemical and histological methods for determining cell division and death are also available. Cell division can be estimated from the number of cells that have incorporated labeled DNA precursors such as bromodeoxyuridine (BrdU) or tritiated thymidine during S-phase of the cell cycle. The frequency of apoptotic cell death can be estimated by counting the number of apoptotic bodies in H&E-stained liver sections [6]. More recently a fluorescent technique has been developed which appears less time-consuming and may be more sensitive than the standard procedure [7].

Cells of EAF show higher levels of proliferation than their normal counterparts. This selective growth advantage is likely to be due to mutational events in one or more growth regulatory genes. In the mouse liver, the Ha-*ras* protooncogene has been identified as a frequent target of mutational activation. The relevance of the activated *ras* protein $p21^{ras}$ as endogenous stimulator of proliferation of initiated cells will be discussed in the following chapter.

3. Role of *ras* mutations during mouse hepatocarcinogenesis

ras mutations in chemically induced mouse liver tumors have first been described by Reynolds et al. [8] and Wiseman et al. [9],

Table 1
Some important characteristics of enzyme-altered liver foci

• Multiple enzyme changes	⇒	indicative of alterations in critical cell regulatory genes
• Monoclonal origin	⇒	number of clones gives some estimate of the initiating activity of test compounds
• Altered growth characteristics	⇒	size distribution of foci allows some estimate of the promoting activity of test compounds
• Precursor relationship to tumors	⇒	quantitative determination of foci allows the prediction of liver tumor risk at very low doses

respectively. To date, more than 2000 mouse liver tumors have been investigated. Approximately one-third of these showed specific base substitutions in codon 61 of Ha-*ras*. Certain strains of mice show higher Ha-*ras* mutation frequencies than others [10,11]. Rat or human liver tumors are very infrequently *ras* mutated.

The question as to whether *ras* mutations occur early or late during carcinogenesis is debated and appears to depend – amongst others – on the target organ. In the mouse liver, mutational activation of Ha-*ras* probably occurs at the level of initiation. This assumption is based on the following findings (for example see [12,13]): (i) mutations can be detected in the 'earliest' enzyme-altered lesions in the mouse consisting of only a few hundred cells; (ii) multiple samples taken from different locations of one and the same (*ras* mutated) lesion generally yield the identical mutational signal; (iii) in experiments where *ras* mutations were detected by allele-specific oligonucleotide hybridization, the signals for the Ha-*ras* wild-type and mutated sequences were generally very similar in intensity; the easiest explanation for this would be that all initiated cells of the lesion harbor mutated and wild-type alleles at a 1/1 ratio; (iv) studies from many laboratories have demonstrated that the types of base substitutions detected in Ha-*ras* mutated mouse liver lesions may vary from carcinogen to carcinogen, i.e. the mutational patterns are carcinogen-specific. Taken together, these data strongly suggest that the mutations in the Ha-*ras* gene were directly induced by the carcinogen during initiation.

Even though the *ras* mutation is produced during initiation, its effects become most evident during the subsequent 'promotional' stage which is characterized by the clonal outgrowth of the (mutated) enzyme-altered cell population. Enzyme-altered cells show higher rates of proliferation than normal hepatocytes, an effect which is likely, although not unequivocally proven, to be produced by the structurally modified $p21^{ras}$ protein encoded by the activated protooncogene. *ras* mutations are (at least with standard procedures) not detectable in normal hepatocytes but are present in up to 50% of mouse hepatomas. It thus appears that the activated $p21^{ras}$ protein drives the proliferation of initiated hepatocytes and confers a selective growth advantage to the mutated cells. Since stimulation of cell proliferation is also a hallmark of tumor-promoting agents, the activated $p21^{ras}$ protein may be termed an 'endogenous tumor promoter'.

4. Action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during rat hepatocarcinogenesis

The effects of exogenous tumor promoters have been most extensively studied in the rat liver system and will be exemplified in the present paper by the action of the dioxin TCDD, which is a potent tumor promoter in rat liver [3]. The effects of TCDD on the proliferation of normal and GST-P-positive hepatocytes were analyzed in an initiation → promotion experiment in the rat. For this purpose, diethylnitrosamine (DEN)-initiated female Wistar rats were chronically treated with TCDD for various periods of time. To determine cell division (BrdU-labeling) and apoptosis, liver sections were prepared and processed for analysis of BrdU-positive hepatocyte nuclei and apoptotic bodies, respectively, as previously described [7]. As shown in Table 2, BrdU-labeling indices (LIs) and apoptotic indices in GST-P-positive liver foci of control animals were ~20-fold higher than in normal hepatocytes. While BrdU-LIs in normal hepatocytes were more or less unchanged or even decreased by TCDD treatment, BrdU-LIs in GST-P-positive liver foci were slightly increased over the respective non-dioxin-treated controls. On the contrary, apoptotic indices were reduced to varying extents by TCDD treatment in both normal hepatocytes and GST-P-positive liver foci. This effect was comparatively weak at the first time point of analysis (31 days), and was most pronounced after 115 days of TCDD treatment, indicating that treatment of animals with TCDD for prolonged periods of time results in a progressive inhibition of apoptosis, which might lead to a selective growth advantage of the enzyme-altered cell population. Moreover, the observation of strongly reduced apoptotic indices along with marginal increases in BrdU-LIs sug-

Table 2
Effect of chronic TCDD treatment on cell division (BrdU-labeling) and apoptosis in GST-P-positive liver foci and normal hepatocytes

Treatment[a]	31 days		73 days		115 days	
	Normal tissue	Foci tissue	Normal tissue	Foci tissue	Normal tissue	Foci tissue
BrdU-labeling index (%)[b]						
Control	0.73 ± 0.36	13.94 ± 1.84	0.56 ± 0.20	10.25 ± 5.36	0.33 ± 0.22	6.79 ± 4.46
TCDD	0.41 ± 0.13	19.19 ± 6.62	0.26 ± 0.08[c]	11.50 ± 3.13	0.40 ± 0.43	9.47 ± 3.80
Apoptotic index (%)[b]						
Control	0.14 ± 0.08	1.64 ± 1.33	0.29 ± 0.20	6.14 ± 5.79	0.32 ± 0.23	6.19 ± 3.93
TCDD	0.10 ± 0.09	1.14 ± 0.97	0.07 ± 0.03[c]	1.38 ± 0.87	0.20 ± 0.08	0.80 ± 0.75[c]

[a] Female Wistar rats were given DEN (10 mg/kg body wt.) by stomach tube for 10 days. After a recovery period of 8 weeks, animals were either treated with TCDD (1.4 μg/kg body wt.; s.c. injections at bi-weekly intervals) for the time periods indicated or served as vehicle controls (corn oil). Groups of animals were killed at 31, 73 or 115 days after start of TCDD treatment.
[b] BrdU-labeling and apoptotic indices were determined as described previously [7]. Values represent means ± S.D. from 4–5 animals.
[c] Significantly different from control ($P < 0.05$; Wilcoxon rank sum test).

gests that inhibition of apoptosis in enzyme-altered liver foci may be more important for the tumor-promoting activity of TCDD than stimulation of cell division.

Birth and death of 'initiated' cells are likely to represent stochastic processes [1]. Under this basic assumption, which is schematically outlined in Fig. 2, we may now consider the consequences of TCDD-mediated decreases in the rate of death of the putatively initiated cells: first, it is obvious that any decrease in the rate of natural cell death will increase the net proliferation rate of the 'initiated' cells (Fig. 1). This partly explains the promoting effect of TCDD and related compounds in rat liver (see also [15]). Second, single initiated cells and small clusters of initiated cells are at high risk of becoming extinct by apoptosis while the probability of extinction exponentially decreases with increasing number of cells per lesion. Consequently, any agent, like TCDD, that inhibits apoptosis will increase the number of surviving clonogenic 'initiated' cells, an effect that may not be easily distinguishable from effects seen with 'classical' genotoxic carcinogens [10].

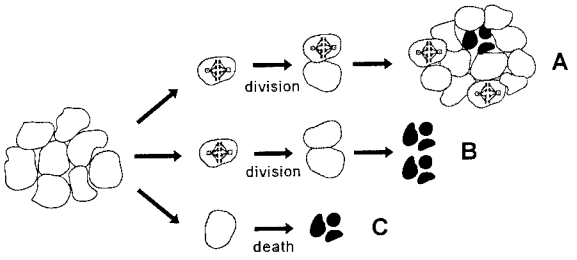


Fig. 2. Implications of cell death on the outgrowth and survival of clones of enzyme-altered cells (modified from [14]). Cell death by apoptosis is frequently observed in larger liver foci (A). Due to the stochastic nature of this process entire clones may become extinct. The probability of extinction is inversely related to the number of cells per clone. Consequently, single initiated cells (C) and small clones (B) are at particularly high risk of becoming eliminated and may therefore never develop into visible EAF.

Dioxins are generally believed to act via complexation to and activation of the Ah-receptor/ARNT-complex which then specifically binds to DNA and transactivates the transcription of responsive genes [16]. Additional TCDD-activated pathways have also been hypothesized by Matsumura [17], who postulates that Ah-receptor activation may initialize a signal cascade through $p21^{ras}$ and the MAP kinase pathway which would ultimately activate genes containing AP1-responsive elements. The involvement of $p21^{ras}$ as a central switch in this pathway is of interest in the context of the present paper since it points towards a similarity of action produced

by aberrant signal transduction by 'endogenous' ($p21^{ras}$) and exogenous tumor promoters.

5. Concluding remarks

The consequence of action of 'initiators', e.g. activation of a protooncogene such as *ras*, is the continual stimulation of proliferation of initiated target cells. The responsible cellular proteins may be regarded as 'endogenous tumor-promoting factors' which operate through cellular signalling pathways that may also be affected by exogenous 'tumor promoters'. On the other hand, inhibition of apoptosis of spontaneously initiated cells by 'tumor promoters' may increase the number of clonogenic initiated cells, an effect that may be termed 'indirect initiating activity'. In light of these considerations chemical carcinogens which are believed to act via distinct mechanisms ('genotoxic' versus 'epigenetic') may ultimately produce overlapping effects, raising some doubts on the practicability of categorizing these compounds into 'initiators' and 'promoters'. In fact, this categorization is purely operational and one could simply argue that pure 'initiators' and 'promotors' are very unlikely to exist. Our critisism, however, goes beyond this point. The terminology 'endogenous promoters' and 'indirect initiators' used above is deliberately provocative to underline this fact, although we are aware of the associated semantic problems. Our argumentation is not intented, however, to question the general validity of the concept of 'initiation' and 'promotion' as such. Indeed, the liver system appears to represent a very valuable tool to investigate the underlying mechanisms of action, namely the nature and consequences of heritable changes occurring in target cells during carcinogenesis and the kinetics of proliferation of the affected cells during the process.

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Asthma and the environment: do environmental factors affect the incidence and prognosis of asthma?

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Abstract

Rising mortality and prevalence rates for asthma in the United States and other countries have again raised concern that environmental agents, particularly air pollutants, may be responsible. While asthma has been linked to environmental causes and triggers, we still have not found specific agents underlying the increasing morbidity and mortality from the disease. Research may be strengthened by the identification of genes for asthma, leading to improved characterization of gene-environment interactions and by more accurate methods for exposure assessment.

Keywords: Asthma; Environment; Indoor air pollution; Outdoor air pollution; Epidemiology

1. Introduction

Asthma is a chronic disease characterized by symptomatic and variable obstruction to airflow within the lungs [1]. It is the most common chronic disease of childhood and it remains a common problem in adults, reflecting the persistence of childhood asthma and the new cases that occur during adulthood. Familial aggregation of asthma is well documented and asthma is considered to have a genetic basis, but interactions between environmental and genetic factors are considered to have a central role in determining the onset of asthma and its clinical course [2]. Definitions of asthma emphasize the variable nature of the airflow obstruction, the presence of airways hyperresponsiveness, and the inflammatory nature of the underlying disease process. While there are no uniformly applied criteria for establishing the presence of asthma, clinicians rely on an appropriate symptom history and usually on the demonstration of intermittent airflow obstruction as well. Epidemiologic approaches to establishing the occurrence of asthma have primarily relied on questionnaire reports of physician diagnoses, characteristic symptom patterns, or appropriate physiological abnormalities, including in recent years the response to airways challenge testing [3].

There has long been concern that environmental factors adversely affect morbidity and mortality from asthma. Epidemic occurrence of asthma in association with environmental exposures has been noted widely and many outbreaks remain unsolved, although some have been linked to sources of exposure within the community. The recent identification of asthma associated with community exposure to soybean

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proteins during unloading of ships provides a reminder of the potential for environmental agents to cause asthma [3]. In the example of soybean asthma, epidemics of asthma in Barcelona, first noted during the 1980s, were linked to days on which soybeans were unloaded at the harbor. Subsequently, an antigen in the beans was determined to be the cause and implementation of appropriate industrial hygiene control measures at a defective silo eliminated this epidemic. Many other environmental factors, to which exposure occurs in both indoor and outdoor air, have been associated with morbidity and mortality from asthma [4].

Beginning in the 1980s, rising mortality from asthma was noted in the United States and a number of other countries [5]. Data for the United States also showed that the prevalence of asthma had increased, suggesting a rising incidence of the disease. Previous rises and falls in mortality from asthma had been documented, but the most recent increase again raised concern as to the underlying causes and environmental pollutants were again among the suspect risk factors [4,6]. In the United States, generally improving trends of outdoor air quality have shifted concerns from outdoor air to indoor air.

The changing patterns of prevalence and mortality lead to two general hypotheses concerning asthma and the environment:

- Have environmental factors increased the incidence and thereby the prevalence of asthma?
- Have environmental factors adversely affected the natural history of asthma and thereby increased mortality?

Recent descriptive data provide a compelling indication of the role of the environment in determining the frequency of asthma and other allergic diseases. Von Mutius and colleagues [7] compared the prevalence of asthma and other allergic diseases in children living in eastern and western Germany. Although the genetic heritages of the two groups of children were similar, the children from western Germany had a higher frequency of allergic disorders, including asthma, while the children from eastern Germany were more likely to suffer from bronchitis. In the United States, prevalence rates and mortality rates from asthma among inner city residents tend to be much higher than nationwide rates and rates among suburban dwellers; it is likely that the recently recognized problem of 'inner city asthma' has environmental causes that lie in the correlates of low socioeconomic status.

This presentation briefly reviews the status of evidence related to the two hypotheses. It begins by considering epidemiologic approaches to investigating asthma and to testing hypotheses concerning the environment and asthma. Limited reviews follow of the evidence on risk factors for the influence of asthma and on the prognosis of asthma and factors influencing asthma's prognosis.

2. Approaches to investigating asthma in the environment

We investigate asthma in the environment using the complementary research disciplines of clinical toxicology and epidemiology. In 'clinical studies', volunteer subjects are exposed to environmental agents of interest in controlled settings. The exposure may take place with intermittent or continuous exercise to increase the delivery of pollutants to the lung. The laboratory setting of such exposures facilitates detailed physiological assessment of responses and invasive methods may be used to obtain biomarkers of response. For example, bronchoalveolar lavage may be conducted after exposure in order to obtain cells and fluid for analysis. Of necessity, controlled exposures are brief and limited to agents at concentrations considered to carry an acceptable degree of risk to volunteers. The findings of clinical studies may be limited by the reliance on volunteer subjects of uncertain representativeness to the population of asthmatics in general and by the exclusion of more severely affected persons with asthma. Additionally, the pollutant exposures used in clinical studies do not usually replicate the types of complex mixtures found in outdoor and indoor environments.

Epidemiological study designs have the strength of investigating the consequences of exposures occurring in the community setting. The full array of study designs has been applied

to the problem of asthma and the environment: ecological studies, cross-sectional studies or surveys, cohort studies, case-control studies, and clinical trials (Table 1). The ecological approach has been used to develop hypotheses concerning asthma and the environment; for example, higher rates of asthma mortality in urban areas led to diverse hypotheses concerning effects of outdoor and indoor pollutants. Numerous cross-sectional surveys have been conducted on asthma. Typically, these studies provide an estimate of the prevalence of asthma and the findings may be used to describe associations between asthma and potential risk factors. However, there is likely to be bias between cross-sectionally observed associations as affected asthmatics modify their environment to improve health status. Fewer cohort and case-control studies have been conducted. Both designs may be informative about factors predicting the onset of asthma and the cohort study is the appropriate design for observing the natural history of asthma. Clinical trials involving randomization of participants to measures intended to reduce risk of asthma or morbidity from asthma, have also been conducted. For example, trials are now underway in infants at high-risk for asthma to learn if environmental modification can reduce the incidence of the disease.

While these observational designs have been informative with regard to asthma and the environment, the findings may be limited by difficult methodologic problems, including establishing the presence of asthma, documenting and estimating exposures to environmental agents, and controlling for the effects of other factors, referred to as confounding factors, which may also be relevant. For some hypotheses of current interest with regard to asthma, notably large populations and valid, but efficient and practicable methods for exposure assessment are needed. We can anticipate the incorporation of genotyping of asthmatics in epidemiological studies in the future, undoubtedly with substantial gain in the capability of testing hypotheses in a more focused fashion but also at greater cost.

3. Environmental factors and the risk of asthma

Asthma is considered to be a multifactorial disease with incidence determined by underlying genetic predisposition and by exposure to environmental triggers [1]; while gene-environment interactions are emphasized in current concepts of pathogenesis, it is clear that there are environmental agents that have the capacity to sensitize individuals and cause asthma, even without identifiable predisposition. For example, toluene diisocyanate can cause asthma following exposures at extremely low concentrations as with may other occupational agents [8]. In addition to genetic background, factors associated with asthma include lower respiratory infections particularly in early childhood, indoor allergens and possibly other indoor pollutants, selected outdoor pollutants, and a large number of occupational agents [2].

A long list of indoor and outdoor air pollutants are suspect causes of asthma (Table 2). Indoor pollutants have received substantial emphasis as causes of asthma [9]. Indoor allergens of concern come from molds and fungi, insects, rodents, and pets; current hypotheses about asthma and allergens emphasize exposures to

Table 1
Epidemiologic study designs

Case-control study: an analytical design involving selection of diseased cases and nondiseased controls followed by assessment of prior exposures
Clinical trial: an analytical design involving random assignment of exposure to two or more subject groups
Cohort study: an analytical design involving selection of exposed and nonexposed subjects with subsequent follow-up for development of disease
Cross-sectional study: subjects are identified and exposure and disease status determined at one point in time
Ecological study: study design involving comparison of disease rates in groups

Table 2
Indoor and outdoor air pollutants relevant to asthma

Outdoor	Allergens
	Particles
	Sulfur oxides
	Nitrogen oxides
	Ozone
	Chemical emissions
Indoor	Allergens
	Tobacco smoke
	Organic compounds
	Nitrogen oxides

allergens from house dust mites, cockroaches, and cats. In a cohort study of high-risk children in England, house dust mite exposure in early life predicted the onset of wheezing as the children were followed up to age 8 years [2]. Cats are also a potent source of allergen, which is persistent in the environment and exposure is widespread as the allergen is carried from location to location on clothing [10].

Environmental tobacco smoke (ETS) is the term used to refer to the mixture of sidestream smoke and exhaled mainstream smoke that contaminates spaces where smoking is taking place. There has long been concern that passive smoking could contribute to the development of asthma. Rising exposure to environmental tobacco smoke in indoor environments is one potential explanation for the increasing frequency of asthma, particularly in inner cities where smoking rates tend to be high. The hypothesis that environmental tobacco smoke exposure causes asthma has been tested in diverse cross-sectional studies and in case-control studies [11]. To date, however, the evidence has not been conclusive and, in its 1992 risk assessment, the U.S. Environmental Protection Agency [11] concluded that passive smoking was associated with asthma but stopped short of characterizing the link as causal.

Other indoor pollutants that might contribute to the causation of asthma include nitrogen oxides from gas cooking ranges and volatile organic compounds, such as formaldehyde, released from furnishings, building materials, and household products. To date, however, definitive evidence on these pollutants as causes of asthma has not been reported.

Investigating outdoor pollutants as risk factors for the onset of asthma is made difficult by the problem of exposure estimation and controlling for potential confounding factors. Allergens and outdoor air are known to exacerbate asthma but have not been shown to cause asthma under usual circumstances. While ecological evidence of varying asthma occurrence has suggested possible effects of outdoor pollutants, confirmatory studies of more definitive design have not been undertaken.

Of course, asthma has a multifactorial etiology and investigations on its etiology need to be designed to either assess a single factor, while rigorously controlling for other factors, or to test the effects of multiple factors in a design that allows a disentangling of their effects. A recent report of a case-control study conducted in Montreal illustrates the latter approach. Infante-Rivard [12] conducted a case-control study of new cases of childhood asthma, identified through hospital emergency rooms. Exposures in the home and family characteristics were contrasted for the cases and controls. The analysis simultaneously tested the effects of indicators of familial predisposition, such as having parents with asthma or siblings with asthma, and of environmental exposures, such as type of heating and level of smoking in the home. The study showed significant effects of family history on asthma risk as well as an affect of parental smoking. Additional studies of this design may prove informative, particularly when combined with a careful assessment of phenotype and genotype.

4. Natural history of asthma and environmental factors

Environmental factors may contribute to morbidity and mortality from asthma by adversely affecting the natural history of the disease. Fortunately, in spite of the attention focused on mortality from asthma, the prognosis of the disease is generally quite favorable. In fact, in a

recent report on the prognosis of 2499 residents of Rochester, Minnesota with a diagnosis of asthma made between 1964 and 1983, having a diagnosis of asthma did not adversely affect survival [13]. In addition to environmental and occupational exposures, other prognostic factors for asthma include age, the underlying severity of the disease and adherence with medication.

In general, asthma is a persistent and chronic disease. Several long-term studies have documented the natural history of childhood asthma well into adulthood [2]. These cohort studies, one involving children from Australia and the other children in the Netherlands, found that most children with asthma were still symptomatic decades later. In the study in the Netherlands, being symptomatic in adulthood was predicted by sex (males having more symptoms than females), by the degree of airways responsiveness in childhood, by having symptoms in childhood, and by becoming a cigarette smoker.

Many studies have now addressed indoor and outdoor pollutants as factors that exacerbate the status of persons with asthma. The research designs employed include the cross-sectional study and short-term cohort studies, often referred to as 'panel' studies. In the latter design, the status of persons with asthma is monitored and variation in status is assessed in relationship to environmental exposures.

Evidence from panel studies has shown that asthma may be worsened by exposure to oxidant pollution and possibly particulate air pollution [14]. These studies have shown short-term associations of outdoor air pollution levels with frequency of symptom reports reflective of exacerbation, need for additional medication, and level of lung function [14]. Over the longer-term, however, air pollution appears to have little impact on mortality from asthma. Lang and Polansky [15] compared death rates from asthma in the city of Philadelphia between 1969 and 1991 with trends of the concentrations of major air pollutants including ozone, carbon monoxide, nitrogen dioxide, sulfur dioxide, and particulate matter. Over these years, the concentrations of these pollutants declined while rates of death from asthma increased. Present trends of rising asthma mortality in many developed countries cannot be attributed to outdoor air pollution.

Panel studies have also indicated possible effects of indoor pollutants and clinical trials show that measures to reduce levels of house dust mite allergen favorably affect the status of children with asthma [10]. Convincing evidence has shown that environmental tobacco smoke causes exacerbation of asthma. In fact, in its 1992 risk assessment, the Environmental Protection Agency concluded "... passive smoking is causally associated with additional episodes and increased severity of asthma in children who already have the disease" [11].

5. Conclusions

Asthma and the environment have been vigorously investigated during recent decades. Cycles of asthma mortality and morbidity have evoked concern about environmental factors that may cause and exacerbate the disease. Evidence from clinical studies and epidemiologic research has successfully identified some environmental factors that contribute to the burden of morbidity and mortality of asthma. However, we still have not identified the cause of the current increase in asthma mortality. Our research may be strengthened by the identification of genes for asthma and more accurate methods for exposure assessment. However, we should be able to gain substantial insights by better applying existing methods to an immediate problem.

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Occupational asthma

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Abstract

Occupational asthma is an important occupational respiratory disorder, both in terms of morbidity, disability and in the total number of cases. The two types of occupational asthma are classified on the basis of their temporal relationship to onset. Occupational asthma with latency reflects allergic occupational asthma and is a condition characterized by a preceding latent period of workplace exposure during which allergic sensitization to a material present at the work site occurs. It is characterized biochemically by immunologic alterations and physiologically by variable and work-related airflow limitation with the presence of both specific and nonspecific airway hyperresponsiveness. In contrast, occupational asthma without latency is an asthmatic condition that develops suddenly and without a preceding latent period, as epitomized by the reactive airways dysfunction syndrome (RADS). RADS is distinguished physiologically by chronic, persistent nonspecific airway hyperresponsiveness and usually occurs after a single brief high-level exposure to an irritant gas, vapor of fume; new information suggests that a more prolonged irritant exposure, in certain susceptible persons with a pre-existing allergic predisposition, can also lead to the initiation of new-onset asthma. The factors that may be influential in the pathogenesis of occupational asthma include: exposure characteristics, industrial factors, job attributes, geographic and climatic conditions, economic considerations and personal or host conditions, such as atopy and cigarette smoking. Preventive measures and opportunities for intervention are essential and must address plans for reducing or eliminating accidents and spills, as well as plans for engineering control methods and proper and effective local exhaust ventilation. Medical surveillance programs are the keystone for prevention and should identify persons who are at an increased risk for developing occupational asthma, as well as detecting asthma at an early stage when intervention options are likely to be successful. For sensitized workers, the best preventive option is completed removal from the work environment.

Keywords: Asthma; Allergic sensitization; Occupational exposure; Bronchial hyperresponsiveness

1. Definition of occupational asthma

A broad definition of occupational asthma ascertains that it is a disorder characterized by episodes of reversible airflow limitation caused by the inhalation of a substance or material that a worker manufactures or uses directly or that is incidentally present at the work site. Another definition is "variable air flow limitation caused by a specific agent in the workplace". This latter definition limits the scope of occupational asthma

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because it necessitates incriminating a specific substance in the workplace. This theme has been echoed by compensation boards which accept a diagnosis of occupational asthma only for a finite number of specific agents. For example, in the United Kingdom, the Industrial Injuries Advisory Council defined occupational asthma as "asthma that develops after a variable period of symptomless exposure to a sensitizing agent at work". In order to qualify, asthma must be due to a predetermined and limited number of agents (platinum salts, isocyanates, epoxy resins, acid anhydrides, colophony fumes, proteolytic enzymes, laboratory animals and insects and grain dust).

Definitions that insist on sensitization exclude non-allergic causes for consideration as occupational asthma. This restriction negates the concept that non-allergic factors can cause occupational asthma, which seems contrary to what is generally appreciated about the pathogenesis of asthma in the general population (i.e., extrinsic-allergic vs. intrinsic-non-allergic asthma). Another classification placed agents into 2 groups: non-specific stimuli (inciters) provoke airway narrowing but do not themselves increase airway responsiveness; and, specific stimuli (inducers) will both provoke airway narrowing and increase non-specific bronchial responsiveness. According to this classification, occupational asthma falls into the second category. The stimuli are able to 'induce' or 'switch on' asthma and non-specific bronchial hyperresponsiveness, either on an allergic or non-allergic basis.

A recently proposed classification seems appropriate and identifies 2 major types of occupational asthma which are distinguished by the presence or absence of a latency period. In this context, occupational asthma due to a sensitizer is a condition occurring after a preceding latent period of exposure when allergic sensitization to a substance or material present in the work site occurs and is characterized physiologically by variable and work-related airflow limitation and the presence of both specific and non-specific airway hyperresponsiveness. At present, there are more than 200 agents documented to cause this type of occupational asthma. In contrast, non-allergic or irritant-induced occupational asthma develops without a preceding latent period. It occurs after an inordinate workplace irritant exposure, and is distinguished physiologically by persistent non-specific airway hyperresponsiveness. In both allergic and non-allergic asthma, pathological changes depict bronchial mucosal injury and inflammation.

2. Factors influencing pathogenesis of occupational asthma

Exposure characteristics can influence the prevalence of asthma. Such factors as chemical type and reactivity, chemical sources and concentration of an exposure are of concern. The intensity of exposure is of particular importance. A single high-level exposure is consequential in the pathogenesis of irritant-induced asthma and intermittent, repeated or persistent exposures are necessary for allergic-type occupational asthma. High level, intermittent exposures have been reported to be important in the pathogenesis of toluene diisocyanate (TDI) asthma; workers with exposure to frequent spills are more likely to report asthma symptoms or show changes in lung function tests. An unmistakable relationship exists between the magnitude of exposure and the prevalence of allergic sensitization. This association has been reported for western red, isocyanates, colophony, baking products, acid anhydrides and cotton dust.

Industrial factors include processes inherent to industrial operations and processes, unique working conditions of the plant or industry, industrial hygiene practices employed in the industry or plant operation, and engineering features of the industrial site. For example, TDI asthma is reported more commonly among workers employed in polyurethane processing than in TDI manufacturing. Hexamethylene diisocyantae (HDI) and TDI display the same vapor pressures and are relatively volatile at room temperature; diphenylmethane diisocyante (MDI) with a lower vapor pressure is not volatile at room temperature. MDI becomes more volatile and likely to lead to asthma when it is heated and vapor pressure increases; this is noted in such

industrial processes as foundry work. The type of industry or process influences prevalence. Asthma develops in about 5% of workers exposed to isocyanates; between 10 and 45% of workers exposed to proteolytic enzymes; workers exposed to grain dust, including millers and bakers encounter asthma prevalence between 2 and 40%.

Job characteristics including type and specific job procedure or practice can influence prevalence. For certain types of jobs or industrial operations, asthma is noted to develop in an exceptionally high percentage of persons exposed. Spray painting with diisocyanates is a particularly hazardous form of exposure since high levels of vapors (HDI, TDI) and particulates (MDI) are airborne. The potential for sensitization is considerable if safety precautions are not followed, such as the use of proper respiratory protective devices. Other potentially dangerous job activities may include pouring, grinding, blasting, sanding, sawing and heating. Unsafe work practices can lead to spills or accidents and resultant high-level exposures.

Geographic and climatic factors can be important. Asthma from red cedar is seen in the western United States. In the Great Lakes area, asthma from grain dusts and flour is frequent. Chemicals are indigenous to many areas, especially the industrial eastern and midwestern sections. Climatic conditions, such as wind direction and humidity may be influential. Environmental exposures have been incriminated in asthma epidemics, such as in Barcelona, Spain where unloading of soybeans caused asthma outbreaks. A variety of factors were explored, including unloading of soybeans in different silos, proximity of silos to urban areas, types of unloading procedures used, presence or absence of bag filters on silos in dust collectors, barometric pressure and wind conditions on epidemic days and air sampling measurements and morphologic examinations of dust collected. All of the analyses led to the conclusion that unloading of soybeans gave rise to sudden, massive release of soybean dust that reached the urban area under appropriate meteorologic conditions and caused the outbreaks.

Economic factors may influence prevalence. An association was observed between the numbers of reported cases of allergic symptoms in workers exposed to western red cedar dust and the quantity of western red cedar imported into Japan. Up to 1960 as little as 10 000 m^3 western red cedar was imported into Japan through the Tokyo and Yokohama-Kawasaki ports. By 1968, imports increased to 440 000 m^3 and were widely distributed among various ports throughout Japan. Coincidentally with the wide distribution of the wood, cases of western red cedar asthma were reported all over Japan.

Personal or host factors are considered to be a key element for asthma development. Atopy is a term which defines a population that distinctly differs with respect to immunologic reactivity and is stipulated to be an important risk factor for occupational asthma. Allergy to the enzymes of *Bacillus subtilis*, platinum salts, animal danders, locusts, or gum acacia are stated to occur more often among atopic workers as compared to non-atopic persons. The relationship between atopy and allergic sensitization is best established for larger molecular weight (>1000 Da) agents compared to those of a lower molecular weight size (<1000 Da). Examples of the former include detergent enzymes, animal handlers allergens, certain insect exposures and bakers. However, there are conflicting data relating atopy and agent molecular size with consequential sensitization to the agent which suggests that other factors play a role in allergic sensitization.

Cigarette smoking appears to predispose workers to allergic sensitization. A connection between cigarette smoking and increased IgE level has been reported. In the general population a higher serum total IgE concentration is noted for smokers compared to non-smokers. Animal investigations indicate that tobacco smoke provokes an increase in serum IgE level and enhances respiratory sensitization to ovalbumin. In the occupational setting, an association between atopy, cigarette smoking and allergic sensitization to a workplace exposure is reported for workers exposed to green coffee bean, tetrachlorophthalic anhydride and platinum salts. A contemplated mechanism to explain the connection between cigarette smoking and allergic sen-

sitization, proclaims that the inhalation of cigarette smoke produces an injury to the bronchial epithelium, possibly widening of the tight junctions between epithelial cells, which enhances bronchial epithelial permeability and leading to heightened penetration of antigen through the epithelial layer; this event leads to more direct access of allergen to the immunologic 'machinery' present in the subepithelium and vasculature.

Workplace airborne irritants may provoke consequences similar to cigarette smoking. Thus, occupational exposures with especially irritating (e.g., isocyanates, red cedar dust, trimellitic anhydride) or enzymatic properties (e.g., subtilisins, esterases, proteases, papain, amylase) are more likely to produce sensitization because their inherent irritant qualities cause bronchial mucosal injury. Industrial operations utilizing these types of agent are potentially dangerous because of the risk of heavy exposures and occurrence of spills and accidents.

Worker turnover is increased in dusty jobs (i.e., foundry work and grain elevators). Bronchial hyperresponsiveness may be a factor in determining health selection out of a dusty work environment. Workers with hyperresponsive airways find it difficult to work in dusty environments and therefore avoid them or quit work early. If workers select themselves out in certain hazardous industries, such as foundry work and employment in grain elevators, such a selection bias may preclude the capability of a study to detect work-related health effects and may actually produce paradoxical inverse associations between exposure and disease.

3. Types of occupational asthma

3.1. Occupational asthma with latency period

This type of occupational asthma encompasses all instances of workplace asthma for which an allergic or immunologic mechanism has been identified and includes most high- and some low-molecular-weight agents. For some agents causing this type of occupational asthma, evidence for an immunologic mechanism is still lacking (or may not exist). While the list of etiologic agents for allergic occupational asthma is over 200 long and growing, only a few of these agents have been extensively studied and characterized with many existing only as anecdotal case reports. One method of classification includes: (1) microbial products (e.g. *Bacillus subtilis* enzymes in the detergent industry); (2) animal, bird, and arthropod products (e.g. urine protein/dander from small mammals in laboratory settings); (3) plant products (e.g. wheat flour in bakeries); and (4) chemicals (e.g. toluene diisocyanate in the plastics industry).

A popular way to classify sensitizing chemicals is as either high-molecular-weight ($\geq$1000 Da) or low-molecular-weight compounds ($\leq$1000 Da). High-molecular-weight compounds are usually proteins, polysaccharides, or peptides of animal, vegetable, bacterial, or insect origin. The prevalence of asthma due to these compounds tends to be high depending on the level of exposure. Atopy seems to be a pre-disposing factor, but not always. These compounds cause asthma by inducing immunologic responses, often characterized by specific IgE antibodies. The pathogenesis of asthma due to high-molecular-weight compounds is no different from that of more common inhalant allergens such as house dust. As a result, occupational asthma due to high-molecular-weight compounds provides a good model for studying extrinsic asthma.

Low-molecular-weight compounds capable of inducing asthma are growing in number. The prevalence of occupational asthma of this type is low and atopy is not a pre-disposing factor. These compounds presumably are too small to act as allergens by themselves. They may elicit immunologic responses by acting as a hapten and combining with a protein carrier molecule to form an allergen. The immune response to a hapten-carrier complex can be directed against the hapten, the carrier, or to a new antigenic determinant. It has been difficult to confirm an IgE-mediated response in many cases of occupational asthma due to low-molecular-weight compounds. TDI asthma appears to encompass a cellular immune response. Skin testing is usually not helpful and specific IgE antibodies are found in only a minority of symptomatic TDI asthmatic

patients. It is likely non-IgE mechanisms are operative in the pathogenesis. Thus, occupational asthma due to low-molecular-weight compounds (such as TDI asthma) may provide a model for studying apparent non-IgE-mediated asthma.

3.2. Without latency period – sudden

In 1985, Brooks et al. reported on 10 individuals who developed a persistent asthma-like illness after a single exposure to high levels of an irritating vapor, fume, gas or smoke. In all cases, asthma developed within 24 h after the exposure and usually within a few hours. Respiratory symptomatology and continued presence of nonspecific airway hyperresponsiveness persisted for years. In 1 person, the persistence of disease was documented to be at least 12 years in duration. The duration of exposure to the incriminated irritant ranged from a few minutes to as long as 12 h. In almost all instances, the exposure was the result of an accident or a situation where there was poor ventilation and limited air exchange in the area. When tested, all subjects displayed a positive methacholine challenge test. There was no identifiable evidence of a pre-existing respiratory complaint in any patient studied; 2 subjects were found to be atopic but in all others no evidence of allergy was identified. The incriminating etiologic agents varied in each case but all were irritants including uranium hexafluoride gas, floor sealant, spray paint containing significant concentrations of ammonia, heated acid, 35% hydrazine, fumigating fog, metal coating remover and smoke inhalation. In 2 cases, bronchial biopsies documented bronchial epithelial cell injury and bronchial wall inflammation; lymphocytes and plasma cells were present but no eosinophilia was observed. Desquamation of respiratory epithelium was seen in one biopsy and goblet cell hyperplasia in another.

A number of subsequent reports recounted cases of reactive airways dysfunction syndrome (RADS)-like disorders from irritant exposures, some displaying atypical features compared to the original RADS description, such as a longer duration of exposure before onset, different physiologic manifestations, not so massive exposure or influences of host factors which had not been described in the original report. The ensuing clinical information on RADS suggested that many investigators were modifying the benchmarks for RADS so that the designation of RADS did not always follow the original diagnostic criteria and that some cases could best be termed as variants of RADS.

3.2.1. Investigation at University of South Florida (USF). An analysis was made of all cases of irritant-induced asthma observed at the Occupational Health Clinic of the USF between June 1989 and December 1992. The aims of the investigation were to: (1) better define the clinical features of irritant-induced bronchial asthma; and (2) examine the role of an allergic predisposition as a host factor in the development of irritant-induced asthma. The retrospective investigation, which identified 86 asthmatic subjects, 65 of whom had workplace (occupational) asthma, was instituted to define prevalence and clinical features of irritant-induced asthma and to assess the developmental role of an allergic predisposition. Of 54 persons (63%) with irritant-induced asthma, 38 (44%) were new-onset asthma in persons without preceding respiratory symptoms or asthma and 16 (19%) were in persons with documented past history of asthma that was clinically quiescent; 11 (12%) were occupational asthma with a latency and caused by a sensitizer in the workplace while 21 (24%) were considered not to be occupational asthma. An 'atopy' or an allergic predisposition was found in 25 of 38 with new-onset irritant-induced asthma (66%) and 15 of 16 with irritant-induced asthma among pre-existing asthmatics (94%); 8 of 21 (38%) were not occupational asthma and 1 of 11 (9%) was occupational asthma with a latency and caused by a sensitizer. Onset time (span of time an exposure continued before patient first developed asthma symptoms) was 'sudden' ($\leq$ 24 h) in 23 of 38 (61%) and 'not-so-sudden' ($\geq$ 24 h) in 15 (39%) with new-onset irritant-induced asthma. A longer onset time correlated with the presence of an 'allergic potential', especially with exposures $\geq$7 days ($P <$ 0.01). The study suggests that there may be 2 clinical types of irritant-induced asthma: onset is $\leq$24 h and no allergic predisposition seems

necessary; and with exposures ≥24 h and where an allergic potential seems consequential. The data suggest that irritant exposure may initiate irritant-induced asthma in susceptible persons, perhaps similar to a respiratory infection or a large allergen exposure. The not-so-sudden type of irritant-induced asthma exemplifies the interaction between environmental and host factors in the initiation of asthma.

4. Opportunities for intervention

Accidental high-level exposures, such as spills, have been reported to have the most serious consequences but provide the greatest opportunity for intervention and development of preventive measures. The planning of procedures to follow in the event of an accident spill is necessary. Engineering controls focusing on proper and effective local exhaust ventilation can reduce worker risks. Other control strategies that may reduce worker exposures include limitation of exposure at the source and installing transmission barriers. Engineering controls require regular maintenance programs and continued evaluation of the industrial process. The use of personal protective devices is generally considered inappropriate, but may be instituted during special situations and generally for only short periods of time. Control options can include the substitution of a sensitizing agent for an agent less likely to cause sensitization. An investigation of the industrial flow schematic may lead to a way of introducing changes in the process which can lower exposure. Various engineering control methods, such as containment, enclosure or isolation may be an appropriate option. Limiting worker exposure time or job rotation may be possible in some special circumstances. Medical surveillance programs are the keystone for prevention and should identify individuals who are at an increased risk of developing occupational asthma, as well as detect disease at an early stage where intervention options are likely to be successful. An occupational health surveillance program may include pre-employment and periodic medical examinations, immunologic monitoring and periodic spirometric surveys. Informing employees about the potential workplace hazards and proper training for safe work practices is of paramount importance. Tests that measure non-specific bronchial hyperresponsiveness should not be used as pre-employment screening tests to exclude potential individuals at risk. There is no decisive evidence at present that pre-existing bronchial hyperresponsiveness represents a risk factor for the development of occupational asthma. It is also inappropriate to exclude atopic persons from employment. Institution of a smoking cessation program is an important option to consider. Specific bronchial provocation studies may be invaluable in certain circumstances, but these studies are indicated for: (1) the investigation of a previously unreported sensitizer; (2) the identification of the precise etiology where a number of compounds may be at fault; or (3) for medical-legal purposes. Specific bronchial provocation studies should only be carried out by experienced personnel in a hospital setting. A number of modifications of the 'stop-resume' work test have been published which can be utilized in suspected cases of occupational asthma. Inclusion of testing for non-specific bronchial responsiveness adds to the specificity of the testing. The demonstration of an increase in responsiveness after returning to work aids in establishing a causal relationship.

For sensitized persons, the best preventive option is complete removal from the work environment because there are reports of fatal consequences of sensitized individuals who continue to work. The early identification of disease, complete cessation of exposure and complete removal from the work environment may allow the eventual resolution of asthma symptoms and non-specific airway hyperresponsiveness. On the other hand, a majority of cases persist after exposure is terminated, as a chronic asthmatic condition associated with non-specific airways hyperresponsiveness. An important observation suggests that the continued exposure of a sensitized individual may lead to a persistent asthmatic condition. A more favorable prognosis is reported to occur with a shorter duration of symptoms before confirming a diagnosis of occupational asthma, maintenance of normal pulmonary function, and the presence of a lesser degree of non-specific airway hyperresponsiveness at

diagnosis. While the best preventive option for a sensitized worker is complete removal from the work environment, modification of this approach, such as improving work-site ventilation to reduce exposure or the use of respiratory protective devices is not medically ethical for an already sensitized individual. Medical therapy to allow a person to better tolerate a workplace exposure is also not generally recommended. However, in situations where the patient cannot or will not avoid continued exposure the medico-ethical issue arises and can only be resolved on an individual case-by-case basis.

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Toxicology Letters

Environmentally induced asthma

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Abstract

Asthma is characterized by inflammation, reversible airway obstruction, and increased airway responsiveness to various stimuli. Despite advances in understanding of the pathophysiology and in developing new treatments, asthma prevalence and mortality have been rising over the last decade, after a steady decline in the 1970s. Risk factors for environmentally induced asthma include air pollutants, tobacco smoke, wood smoke, and excessive allergen exposure. In controlled human chamber studies, asthmatics demonstrate increased susceptibility to outdoor pollutants such as sulfur dioxide, nitrogen dioxide, and acidic particles with acute reductions in lung function during and following exposures; responses are enhanced by increased ventilation, for example during exercise, or breathing cold air and/or dry air. The evidence is even stronger that inhaled indoor allergens have a causal relationship to asthma. It is possible that changes in housing conditions have led to increased levels of dust mite and other proteins in homes with consequent increases in the prevalence of sensitization. Avoidance of specific allergens such as house dust mite over months results in a reduction in clinical symptoms and bronchial hyperresponsiveness. The interaction between aeroallergens and air pollutants in triggering environmentally induced asthma is an area of active research.

Keywords: Asthma; Air pollution; Aeroallergens; Environmental controls

1. Introduction

Asthma is characterized by inflammation, reversible airway obstruction, and increased airway responsiveness to various stimuli. Despite advances in understanding of the pathophysiology and in developing new treatments, asthma prevalence and mortality have been rising over the last decade, after a steady decline in the 1970s. It is not entirely clear why rates of death have been higher among blacks than whites, but there is evidence of a much higher prevalence of asthma among blacks than whites [1]. Risk factors for environmentally induced asthma include air pollutants, tobacco smoke, wood smoke, and excessive allergen exposure.

This paper addresses the issue of environmentally induced asthma in the context of the patient. First, it reviews the health effects of criteria air pollutants focusing on controlled human exposures of asthmatics. Next the epidemiology of aeroallergens and asthma is addressed; we briefly examine the role of environmental controls for 3 common allergens: house dust mite, cat, and cockroach. Finally, we consider interactions between criteria pollutants and aeroal-

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lergens and emphasize the need for further investigation in this area.

2. Air pollutants and asthma

There are large numbers of compounds in the ambient air which are highly toxic at certain concentrations. The likelihood of an adverse response to an inhaled pollutant depends on the degree of exposure to the pollutant and individual characteristics of the exposed person that determine susceptibility. The concept of susceptibility is highly relevant to public health protection and the delivery of health care. Human responses to environmental agents that affect the respiratory system depend on many factors, including genetic differences in the way agents are metabolized, difference in target sites in the lung and airways, and the defense mechanisms of the respiratory system. Also important are preexisting diseases, airway reactivity, age, gender, pregnancy, and nutritional status [2]. Exposures to other agents such as cigarette smoke or combinations of environmental agents may also influence susceptibility to certain air pollutants.

2.1. Asthmatics as a susceptible group

The U.S. Clean Air Act (CAA) mandated that the National Ambient Air Quality Standards for the criteria pollutants (e.g., ozone, NO_2, SO_2, particles) were to be set low enough to protect the health of all susceptible groups within the population. Two diseases, asthma and emphysema, were identified specifically in the CAA as associated with increased susceptibility.

The term 'susceptible' has been most often applied to groups who share one or more characteristics that place them at increased risk compared with people without these characteristics (Table 1). For asthmatics and individuals with chronic obstructive pulmonary disease (COPD), airway reactivity and reduced levels of lung function have been identified as mechanisms of increased susceptibility. Controlled laboratory studies of these groups have attempted to identify specific effects of pollutants as assessed primarily by pulmonary mechanics.

Since the epidemiological studies examining exacerbations of asthma and increased levels of specific pollutants have recently been reviewed in detail [3], this paper will focus on findings from controlled laboratory studies. In brief, the epidemiology has linked acute decrements in lung function, increased medication use, and increased emergency room visits with ozone and particles. To date, the controlled clinical studies have identified asthmatics as susceptible to SO_2, sulfuric acid aerosols, and perhaps NO_2, whereas individuals with COPD may have increased responsiveness to NO_2. For example, the most striking effect of acute exposure to SO_2 at concentrations of 1.0 ppm or below is the induction of bronchoconstriction in asthmatics after exposures lasting only 5 min [4]. In contrast, inhalation of concentrations in excess of 5.0 ppm causes only small decrements in airway function

Table 1
Groups considered susceptible to inhaled air pollutants

Populations	Potential mechanism	Health effects
Infants	Immature defense mechanisms of the lung	Increased risk for respiratory infection
Elderly	Impaired respiratory defenses, reduced functional reserve	Increased risk for infection, increased risk for clinically significant effects on function
Asthmatics	Increased airway responsiveness	Increased risk for exacerbation of respiratory symptoms
Cigarette smokers	Impaired defense and clearance, lung injury	Increased damage through synergism
Individuals with IHD	Impaired myocardial oxygenation	Increased risk for myocardial ischemia
Individuals with COPD	Reduced level of lung function	Increased risk for clinically significant effects on lung function

IHD, ischemic heart disease.
COPD, chronic obstructive pulmonary disease.

in normal subjects. Similarly, clinical studies have identified exercising adolescent asthmatics [5] as susceptible to sulfuric aerosols at high ambient concentrations, levels that do not affect healthy volunteers. Although several controlled human studies have found asthmatics responsive at low levels of NO_2, the findings have not been consistent [6]. The conflicting results among these studies are probably related to differences in subject selection and exposure protocols. Rather surprisingly, asthmatics and healthy volunteers have shown nearly equivalent responses in terms of pulmonary decrements to ozone. Several possible explanations exist. In contrast to studies with healthy volunteers, studies of asthmatic subjects have not been performed using multiple-hour exposures or repeated daily exposures. Furthermore, few studies with asthmatic subjects have incorporated multiple periods of exercise, an essential factor in provoking changes in airway function with low-level ozone exposure in healthy volunteers. Thus, the issue of ozone sensitivity will not be resolved until populations of normal and asthmatic subjects are compared using protocols of similar design.

3. Aeroallergens and asthma

Allergens are clearly an important precipitant of acute asthma. A brief interview with any cat-sensitive asthmatic makes this relationship dramatically clear. The connection between aeroallergens and chronic asthma is much more difficult to demonstrate. Nevertheless, there is now considerable data implicating aeroallergens as the cause of chronic asthma in many patients. The case for house dust mites in the etiology of asthma is particularly well developed. The argument for causality is based on the Hill criteria which were originally proposed to establish a causal association between a variety of human diseases and cigarette smoking. The data showing that house dust mites fulfill the Hill criteria as a cause for asthma have recently been review by Sporik et al. [7] and will not be reviewed here. Instead, the questions that will be addressed are: (1) what is the epidemiology of aeroallergens and asthma, that is, how does the importance of individual aeroallergens vary in different geographic regions and in different demographic groups of patients? (2) what environmental controls are effective in eliminating aeroallergens? (3) how might pollutants influence the response to aeroallergens? Recognizing that these questions are still areas of active research, our current understanding will be reviewed.

3.1. Epidemiology of aeroallergens and asthma

A variety of allergens are important in asthma including indoor allergens such as house dust mites, pets, cockroaches, and rodents and outdoor allergens such as pollens and spores. In some areas seasonal epidemics of asthma occur because of outdoor allergens. For example, in California there is a seasonal peak in emergency room visits for asthma that coincided with the peak in grass pollen [8]. Naturally, the importance of specific outdoor allergens varies considerably with location. For example, ragweed is an important cause of seasonal asthma in North America but it is unimportant in Europe except for a region in France where it has become endemic. Conversely, olive trees are a major seasonal allergen in parts of Europe but not in the Americas.

Recent studies have shown that indoor allergens important in asthma vary with location and socio-economic class. Examples of this variation include:

3.1.1. Coastal vs. inland locations. Some of the most interesting and rigorous epidemiological studies have been done in Australia by a group led by Anne Woolcock. These investigators have performed large scale investigations involving thousands of elementary schoolchildren in different regions. Children in the same grade were interviewed, tested for immediate hypersensitivity to aeroallergens, and had pulmonary function tests done including histamine bronchoprovocation. Asthma was defined as those children having both a history of wheezing and airway hypersensitivity to histamine. A relative risk for asthma based on sensitization to various aeroallergens could then be calculated. Australia has a

very humid coastal region and becomes progressively drier inland. Thus, exposure to aeroallergens is likely to change depending on how far inland the region is located. Indeed, in the coastal city of Lismore the concentration of the mite allergen Der p I in samples of house dust averaged 83.0 μg/g dust whereas in the inland city of Moree, mites averaged 11.2 μg/g [9]. In Lismore the relative risk for asthma for individuals sensitized to house dust mites increased 21-fold. In Moree mite-sensitization was associated with a 3-fold increase in relative risk for asthma. Conversely, sensitization to *Alternaria* was associated with a 3-fold increase risk for asthma in Lismore but a 6-fold increase risk in Moree. Thus, the role of house dust mite decreased and the role of mold spores increased in the relatively drier inland region. In addition, when the airway sensitivity in the 2 regions was compared, the mite-sensitive children in Lismore had significantly greater airway sensitivity than the mite-sensitive children in Moree, thus establishing a dose-response relationship between the amount of mite allergen in a community and airway hyperresponsiveness of sensitized children. An alarming result from the findings of these workers that may bear on the current increase in asthma in the United States is that there was a 3-fold increase in the prevalence of asthma among 8–10-year-old schoolchildren in Belmont over the 10-year period from 1982 to 1992 [10]. Coincident with this increase in asthma there was a 5.5-fold increase in the concentration of mite allergen in house dust. This dramatic change in asthma prevalence in association with an equally dramatic change in levels of house dust mite strongly suggests but does not prove that changes in levels of aeroallergens may be casually related to changes in asthma prevalence and severity.

3.1.2. Altitude vs. sea level. House dust mites also do not do well at high altitudes. In Briancon the concentration of Der p I was 0.36 μg/g while in Martigues along the Mediterranean Der p I was 15.8 μg/g [11]. In Briancon sensitization to mites is low and is not associated with asthma. Moreover, the prevalence of asthma in Briancon is only half that of Martigues.

3.1.3. Underdeveloped vs. developed regions. A very low prevalence of asthma has been found in some Third World areas. For example, when Highland villages in New Guinea were surveyed in the 1960s the prevalence of asthma was only 0.1% of the adult population. More recent surveys have found that the prevalence of asthma in some of these villages had increased to 7% of adults over the course of about 20 years [12]. This change appears to be related to infestation with house dust mites. In those villages with a high prevalence of asthma the density of mites was 1371/g dust while in those villages where the prevalence of asthma was still low the density of mites was 283/g dust. In addition, villagers with asthma had high levels of mite-specific IgE by radioallergosorbent testing (RAST) compared to individuals in the same village without asthma or villagers from regions with a low prevalence of asthma. The development of mite infestation was thought to have been related to the introduction of cloth bedding.

3.1.4. Inner city vs. suburban areas. Inner city and suburban asthma were compared in Atlanta [13]. Suburban asthmatics were sensitive to cat but not cockroach while inner city asthmatics were sensitive to cockroach but not cat. Sensitivity to house dust mite was similar in both groups.

3.1.5. Asthma in the inner city poor. Poor inner city children have been the subject of a multicenter study of asthma. One surprising result was that sensitization to house dust mites was relatively uncommon in these patients. Indeed, significant levels of house dust mite allergen were found in only 6% of these homes [14]. Instead the major allergen was cockroach. The reason for the paucity of mites in these households has not been established but may be related to greater extremes of temperature in these dwellings.

Levels of exposure are important in sensitization and development of asthma. Even within one geographic area there are dramatic differences in the levels of allergens. For example, in Berlin the level of house dust mite antigen on mattresses varies over a 1000-fold range [15]. This variation is an important determinant of sensitization to mite, that is, the homes of mite-

sensitive atopic children had significantly higher levels of mite allergen than atopic children who were not sensitized to mites. Moreover, a prospective study of children born to atopic parents in Poole, England found that children exposed to high levels of house dust mite in the first year of life developed asthma at an earlier age than infants exposed to lower levels [16].

3.2. Environmental controls

In patients sensitized and exposed to specific aeroallergens, environmental controls can be effective. Three of the most important indoor aeroallergens are considered below.

3.2.1. House dust mites. Moving patients to areas with low levels of mites has been effective in reversing airway hypersensitivity in mite allergic asthmatics. For example, Dutch schoolchildren moved to a sanitorium in Davos or Londoners moved into hospital rooms had progressive reductions in airway responsiveness to histamine [17]. With strict environmental controls for house dust mite, dramatic reduction in symptoms and medication use were seen in a controlled study in Canada in 1983 [18]. This early study did not measure levels of mite allergen or bronchial airway responsiveness. However, a recent German study showed that both of these parameters improved over a 6-month period. This positive result was seen despite the fact that the interventions used were not as comprehensive as those in the earlier Canadian study. Environmental controls for house dust mite have been reasonably well worked out:

(1) Use impermeable covers on mattress and pillows.
(2) Wash all bedding in hot (>130°F) water.
(3) Remove carpets from the bedroom and family room.
(4) Treat unremoved carpets with benzyl benzoate or tannic acid.
(5) Replace or treat cloth furniture.
(6) Reduce indoor humidity, e.g. air-conditioning during summer.
(7) Use filters on vacuum cleaners.
(8) Do not use air cleaners. (Mite allergen is mainly in large particles that settle rapidly.)

3.2.2. Cat. The major cat allergen, Fel I, is produced in both the salivary and sebaceous glands and accumulates in fur. Cat allergen remains airborne for long periods of time due to its presence in small particles. Removing the cat results in a gradual decrease in airborne Fel I. Airborne cat allergen can persist for 6 months and may remain in reservoirs, such as mattresses, for years. Removing the cat must be strongly recommended but this recommendation is often unacceptable. A combination of washing the cat weekly, removing carpets and other reservoirs, and using a room air cleaner can significantly reduce allergen levels [19]. However, the clinical efficacy of environmental controls for cat allergen when the cat is not removed is still not clear.

3.2.3. Cockroach. Cockroach allergen may be the most important indoor allergen for poor, inner city children. Control measures for cockroach allergen have not been studied sufficiently to make any recommendation. Moving out of an infested apartment house may be the only effective option.

4. Interactions between pollutants and aeroallergens

The direct effects of pollutants on the airways have been discussed previously. In addition, there is reason to be concerned about the interaction between pollutants and aeroallergens. There are data that suggest that pollutants may enhance sensitization to aeroallergens and that the combination of aeroallergen and air pollution may result in enhanced bronchoconstriction. Most of the argument for an association between pollutants and sensitization to aeroallergens comes from epidemiological studies and animal experiments. For example, IgE sensitization to red cedar pollen in Japan is higher in polluted urban areas than in forested, rural areas. It was hypothesized that particulate material from diesel exhaust acted as an adjuvant for pollen-specific IgE production and this was confirmed in rat experiments. There has been no direct test of this adjuvant effect in humans.

Recently, limited experimental data in humans have demonstrated that pollutants can increase the airway response to allergen challenge. Using an innovative approach to study environmental interactions, Molfino et al. [20] investigated whether inhalation of 0.12 ppm ozone for 1 h potentiates the airway allergic response in asthmatics with seasonal symptoms. Although ozone did not significantly alter baseline function, reactivity to inhaled allergen was significantly enhanced by prior ozone inhalation. Using a variety of markers ozone has been shown to increase airway permeability; it is conceivable that prior ozone exposure increased access of allergen to subepithelial mediator secreting cells. Despite these intriguing initial observations of interaction, more recent studies have had difficulty confirming the ozone findings. Subsequently, nitrogen dioxide, at concentrations encountered in the home environment, has been shown to potentiate the specific airway response in mild asthma to house dust mite antigen, although both the effect and study populations were small [21]. It is clear that a fertile and important area of research is that of mixtures including 2 or more pollutants, either combined or sequential, as well as combinations of pollutants and allergens. Perhaps an even more exciting area is the potential role of pollutants in enhancing sensitization, e.g., IgE production, in response to inhaled allergen.

5. Conclusions

The environmental effects on asthma are complex. One needs to consider not only pollutants generated by a variety of processes but also the ecology of aeroallergens. This complexity means that careful consideration of the peculiarities of each local environment is critical for optimal management of patients and for establishing public health policies.

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Recent epidemics of poisoning by pesticides

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Abstract

The mechanisms of production of collective poisoning by pesticides have been: contamination of foods consumed by man, cutaneous contact and occupational exposure. It is possible to distinguish 4 groups of alimentary epidemics: (1) contamination during transport or storage; (2) ingestion of seed dressed for sowing; (3) use of pesticides in food preparation because of their organoleptic similarity to alimentary products; (4) presence of pesticides in water or food owing to unsafe use of pesticides. Occupational exposure affects clusters of workers in pesticide manufacture, pesticide application and crop management. Some episodes of every kind of the mentioned groups have been published recently.

Keywords: Pesticide poisoning; Toxic outbreak; Toxic epidemiology

1. Introduction

We have defined toxic epidemics as a pathological condition characterised by a group of symptoms affecting a particular population, which has been exposed for a certain period of time to a chemical product of previously known or unknown pathogenicity [1]. They have been caused by organic or inorganic compounds, natural or synthetic, the presence of which in the environment is often unsuspected and which are of a nearly infinite number.

Many chemicals have produced collective poisonings over the last 50 years. The most frequently implicated among them are the broad family of substances used as pesticides probably due to their high toxicity and widespread use.

The pesticides which have produced toxic outbreaks belong to every family of synthetic products.

The most important mechanisms of production of collective poisoning by pesticides have been: accidental contamination by pesticides of foods consumed by man, contamination of clothes or other vehicles for cutaneous contact and occupational exposure.

It is possible to distinguish 4 groups of alimentary epidemic: (1) contamination during transport or storage; (2) ingestion of seed dressed for sowing; (3) use of pesticides in food preparation because of their organoleptic similarity to alimentary products; (4) presence of pesticides in water or food owing to misuse near harvesting time, misuse of containers, contamination of groundwater and use of excessively high doses in agriculture.

Epidemics by cutaneous contact have affected mainly children populations through clothes or

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diapers contaminated in their transport or storage or in the manufacture procedure.

Occupational exposure affects clusters of workers in 3 typical situations: pesticide manufacture (synthesis or formulation), pesticide application and crop management.

In spite of the preventive measures that have been used to avoid these outbreaks, some episodes of every kind of the mentioned groups have been published in the last 10 years.

2. Food-borne epidemics

We will now analyse the main characteristics of the alimentary epidemics and the evolution of their presentation in the last decades.

Epidemics caused by contamination of food during transport have occurred periodically since 1956.

Contaminated foodstuffs are usually powders, such as flour and sugar coming into contact with the toxic agent when the alimentary product is transported or stored together with the pesticide or in a place previously contaminated by the toxic agent.

The toxic agent is often an insecticide with a low LD_{50}: among 16 episodes (Table 1) in which contamination had occurred by this mechanism, 10 were caused by parathion, 5 by endrin and 1 by carbophenothion. These kinds of episodes involve highly toxic substances that can produce their effects at low doses.

The populations affected are usually large, involving at least a number of families usually living near the source of distribution of contaminated food, such as a bakery.

The latency period is relatively short, from a few minutes to a few hours. As the evolution of such outbreaks is rapid and the characteristic symptoms of exposure to the toxic product appear quickly, a correct diagnosis can soon been achieved on the basis of clinical and epidemiological observations, with analytical confirmation in most cases.

One of the most characteristic episodes happened in Saudi Arabia and Qatar in 1967 when 2 freightloads of flour were contaminated when they were transported beneath loads of endrin causing 4 outbreaks. Altogether 874 people were admitted to hospital and 26 died [2].

The control measures adopted for international transport have been effective in order to prevent these episodes. Thus epidemics originating from international transport ceased in the

Table 1
Epidemics caused by contamination of food during transport or storage

Toxic agent	Contaminated product	Time	Ill	Deaths	Country	Year
Endrin	Flour	2 days	159		England	1956
Parathion	Flour	1 day	200	8	Egypt	1958
Parathion	Flour	1–2 months	360	102	India	1958
	Sugar					
Parathion	Barley	1 day	53	9	Malaysia	1959
Parathion	Flour		29		Yugoslavia	1961
Parathion	Flour	15 days	88	10	Guyana	1962
Endrin	Flour	1 day	3		Egypt	1966
Parathion	Flour		165	63	Colombia	1967
	Sugar					
Parathion	Flour	2 days	559	16	Mexico	1967
	Sugar					
Endrin	Flour	2 days	183	2	Saudi Arabia	1967
Endrin	Flour	1 month	691	24	Qatar	1967
Carbophenothion	Flour	2 days	7		USA	1968
Parathion	Flour			18	Jamaica	1968
Parathion	Flour	22 days	79	17	Jamaica	1976
Endrin	Sugar	2–3 months	194	19	Pakistan	1984
Parathion	Flour	10 days	49	14	Sierra Leone	1986

1960s, however, similar episodes are now found in relation to national transport, especially in developing countries, where such regulations do not exist.

The latest epidemics in Pakistan in 1984 [3] and Sierra Leone in 1986 [4], were caused by 2 well-known toxic products – endrin and parathion.

Between 16 and 26 July 1984, 7 residents in Talalang city in Pakistan, without a prior history of seizure pathology, had convulsions and 2 of them died. From July to September acute convulsions occurred in residents of 21 villages affecting 194 persons; 19 died (9.8%). Endrin was detected in the blood of 12 of 18 patients with a history of convulsions and the possibility that a shipment of food was contaminated with endrin en route to the city has been stressed by the report of the incident. There was evidence that truckers transported any combination of goods, including chemicals and food.

In Sierra Leone in 1986 3 outbreaks occurred between 20 May and 1 June in Kenema and Lalehun, resulting in 49 cases of poisoning and 14 deaths. A detailed epidemiological inquiry based on clinical data and subsequent analytical confirmation showed that 10–15 ml parathion had dripped into a 22.5-kg bag of flour during transport between the mill and a general store in Kenema.

The deviation of seed dressed for sowing for human use has resulted at least in 12 episodes (Table 2): 8 were due to organic mercury fungicides, 1 to hexachlorobenzene, 1 to thallium sulphate and 2 to endosulfan. All but 2 took place between 1955 and 1971. Their severity has not been related to parameters of acute toxicity but to the ingestion of low but continuous doses which resulted in progressive accumulation of the toxicant. They include the Porphyria cutanea Tarda epidemic due to ingestion of seed treated with HCB in Turkey with more than 10 000 people affected (1956–1969) and a 2–10% annual mortality rate [5], and the epidemic caused by the ingestion of seed treated with methyl mercury in Iraq in 1971 with 6530 affected and 479 deaths [6].

The last episode of this kind took place on 15 March 1991 in Sudan [7]. Three hundred and fifty people were affected and 31 people died when a number of villagers attending a funeral ate bread made from maize flour treated some years earlier with endosulfan as a poisonous bait for birds. Samples of bread showed levels of endosulfan of 3776 mg/kg. The origins of the poisoning was traced to a preparation of maize

Table 2
Epidemics caused by seed dressed for sowing or as bait

Toxic agent	Product	Contaminated time	Ill	Death	Country	Year
Thallium sulfate	Barley Flour	12 days	31	6	USA	1932
HCB	Wheat	5 years	3–5000	10%	Turquía	1955
Ethyl mercury	Wheat	6 months	200	70	Iraq	1955
Ethyl mercury	Wheat	6 months	1000	200	Iraq	1959
Ethyl phenyl mercury	Wheat	3 months	100	9	Pakistan	1961
Methyl mercury	Wheat	4 months	45	20	Guatemala	1966
Ethyl mercury	Maize		144	20	Ghana	1967
Methyl mercury	Grain	15 days	4		USA	1969
Mercury	Pig meat Seed Flour		100		Pakistan	1969
Methyl mercury	Wheat Bread	6 months	6530	459	Iraq	1971
Endosulfan	Sorghum Bread	1 day	87		Sudan	1988
Endosulfan	Maize Flour	1 day	350	31	Sudan	1991

treated with 50% endosulfan which was recommended in an anti-bird campaign in 1983. A certain amount of this maize was stored till March 1991 when it was sold for human consumption. A similar case had occurred in Um Badda in 1988. Pesticide-coated sorghum was served as bread at another funeral resulting in the acute poisoning of 87 persons. The sacks were clearly marked in English as contaminated with pesticides but the mill owner was illiterate. Both cases have shown that the optimistic point of view on the disappearance of these episodes was premature, especially with reference to developing countries once again.

In the group of epidemics caused by accidental use of pesticides in food preparation because of their similarity to nutritional products there are 2 sources of error: similarity between the 2 products and wrong packing, labelling or storage. At least 23 episodes have been described (Table 3). The mechanism by which such episodes occur results in the exposure of smaller population groups to the episodes analysed above, varying from 5 to 280 people. The units consist of families or other groups living around a common site of food manipulation – army canteens, hospitals, restaurants, cafeterias. Some of them are not very important from the quantitative point of view but, as a whole, they draw a good picture of the way those toxic products come into contact with food substances and their effects on human beings.

The foodstuffs most frequently confused with pesticides of similar appearance are flour, sugar and salt, and the pesticides most frequently implicated are those with a salty appearance: sodium fluoride and barium carbonate, but there have been problems with insecticides such as lindane, fonofos, methomil and DDT.

The storage of toxic products in glass bottles or plastic bags with no identifying label in kitchens or pantries has been the decisive factor in most episodes.

The worst episode occurred in 1942 in a hospital in Oregon, USA, where sodium fluoride, kept in the kitchen as a cockroach killer, was used instead of powdered milk to make scrambled eggs for the patients. A total of 263 people

Table 3
Epidemics caused by error

Toxic agent	Product	Contaminated time	Ill	Deaths	Country	Year
Fluoride	Pancakes	1 day	7	1	USA	
Fluoride	Cakes		7		USA	
Fonofos	Pancakes	1 day	5			
Fluoride	Cakes	1 day	7		USA	1931
Fluoride	Cakes	1 day	14	2	Germany	1935
Fluoride	Water		21	3	USA	1936
Fluoride	Pudding		141		Germany	1937
Fluoride	Pudding		69		USA	1939
Fluoride	Pancakes		40	12	USA	1940
Fluoride	Eggs	1 day	263	47	USA	1942
Barium carbonate	Tarts	2 days	85		Iran	1945
Fluoride	Flour	1 day	34		Germany	1946
Fluoride	Flour		5	1	USA	1947
DDT	Meat ball	1 day	8		Taiwan	1952
Fluoride	Sugar				Germany	1953
Fluoride	Pears	1 day	280		Germany	1958
Lindane	Dessert	1 day	5		Australia	1959
Fluoride	Cakes		60	1	USA	1961
Barium carbonate	Sausage	2 days	100		Israel	1963
Lindane	Coffee		11		Bulgaria	1965
Methomil	Roti	1 day	5	3	Jamaica	1977
Arsenic		1 day	8		USA	1979
Barium carbonate	Flour	1 day	13	1	Taiwan	1989

had symptoms such as nausea, vomiting and diarrhoea, with blood in the vomitus and stools, followed by general collapse, paralysis and muscular spasm; 47 people died. In spite of the minimal latent period, the identity of the toxic agent was not established until 22 h later.

The last published episode of this kind occurred in Taiwan in 1989 at a family party [8]. Nine out of 13 victims developed nausea, vomiting, abdominal colic, diarrhoea and numbness of the face and distal extremities 1–2 h after ingesting fried flour-coated sweet potatoes contaminated with barium carbonate. One person died and 2 others showed ventricular tachycardia followed by cardiac arrest and respiratory paralysis but were resuscitated. The most prominent sign was acute hypokalemia. Apparently the person who prepared the dessert may have mistaken the barium carbonate for flour. Analysis of sweet potatoes and flour revealed 25.52 and 688 mg/g of barium carbonate.

Last May a new episode broke out in Spain. Three members of a family became ill and died after eating some fish coated with malathion confused for bread. All 3 showed nausea and vomiting, pinpoint pupils, coma Glasgow 3, sweating, hypotension and convulsions. Analytically a severe depression of plasma cholinesterase was found (below 10% activity). One of them died before admission and the other 2, treated with atropine and pralidoxime, developed severe ARDS and multiorganic failure dying 2 and 9 days after the poisoning.

The fourth group of alimentary epidemics was produced by presence of pesticides in water or food due to misuse near harvesting time, misuse of containers, contamination of groundwater or use of excessively high doses in agriculture (Table 4). They are indicators of the widespread and massive use of pesticide products resulting in their presence in drinking water and food. Their origins are not easy to trace and they are the most modern and the most difficult of toxic poisonings to control. The numbers of affected people have varied widely depending on the source of contamination.

Between 1977 and 1988 five outbreaks of foodborne poisoning with aldicarb and its metabolite, aldicarb sulfoxide, were reported in the USA: 3 due to the contamination of hydroponic cucumbers and 2 due to the consumption of watermelons [9,10].

The largest episodes occurred in California, Oregon, and Washington, USA, and Canada in

Table 4
Epidemics caused by environmental contamination and unsafe use of pesticides

Toxic agent	Product	Contaminated time	Ill	Deaths	Country	Year
Toxaphene	Vegetables	1 day	3		USA	1951
Toxaphene	Vegetables	1 day	4		USA	1951
Dieldrin	Flour, salt	4–5 days	21		Germany	1961
Arsenic	Water	2 months	11		USA	1972
Fluoride	Water	1 day	213		USA	1974
HCH	Rice, wheat	2 years	268	4	India	1976
Aldicarb	Cucumbers	15 days	9		USA	1977
Fluoride	Water	1 day	34		USA	1978
Aldicarb	Cucumbers	10 days	5		USA	1978
Arsenic	Water	3 days	8	2	USA	1979
Mercury	Food, water		7		Kenya	1980
Fluoride	Water	1 day	22		USA	1980
Parathion	Oil	1 day	25	18	Senegal	1983
Aldicarb	Watermelons	3 months	1350		USA	1985
Aldicarb	Watermelons	1 day	4		USA	1987
Methamidophos	Cabbage		100		Taiwan	1987
Methamidophos	Cabbage	1 week	64		Taiwan	1987
Aldicarb	Cucumbers	2 months	9		USA	1988
Endosulfan	Cheese		167	2	Sudan	1988

1985, and were caused by the consumption of watermelons contaminated with aldicarb [11]. There were 1376 reported cases with symptomatology congruent with carbamate poisoning.

All these episodes occurred in spite of the fact that the use of aldicarb is forbidden in the US in certain crops like watermelons because it is incorporated into the flesh of the fruit. The source of aldicarb contamination has not been determined but is probably due to their inadvertent or illegal application.

Another aldicarb outbreak was reported in the Irish news media in 1992. Following this report about 30 people had fallen ill at the end of May after eating contaminated cucumbers. Aldicarb is used in Ireland to control insect infestation in potatoes, beans and sugarbeet but not approved for use with cucumbers.

From the chronological point of view (Fig. 1) you can see that group I has experienced a clearcut decrease since the late sixties after the outbreaks in Saudi Arabia and Qatar in 1967 when control measures were adopted for international transport. As we have shown the last episodes have broken out at the national level and can still be a problem in developing countries where it is difficult to implement legislation. Epidemics belonging to group II have decreased thanks to the ban on the higher toxic fungicides to preserve grain. The third group shows also a clear reduction which is not so accurate as the former because these kinds of episodes, affecting few numbers of people, are probably under-reported. As we have stated before, the fourth group is the most modern with 16 episodes reported after 1970.

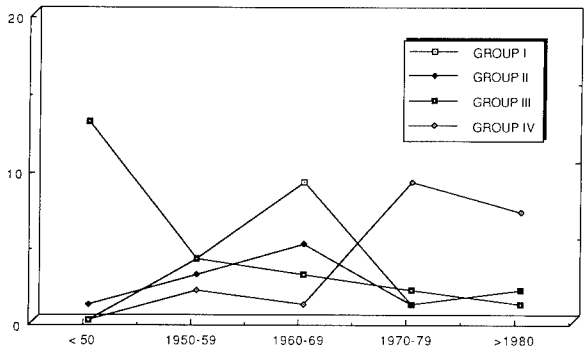


Fig. 1. Chronological evolution.

3. Cutaneous epidemics

We have found 5 episodes of pesticide collective poisoning caused by cutaneous contact with the toxic product or with some vehicle contaminated with pesticides out of the occupational field. Two of them have broken out after 1980 (Table 5).

The first of them occurred in Vietnam in 1981 [13]. It was reported as a haemorrhagic syndrome of sudden onset with signs of neuromeningeal involvement. Two months after the beginning of the epidemic, 741 cases had been detected, with 177 deaths. The origin of this syndrome was the use of talc contaminated with a dicoumarin-type anticoagulant. Warfarin was identified in 54 samples. Accidental confusion with a rodenticide was one of the causal hypotheses.

The last cutaneous episode described was investigated by epidemiologists from the New

Table 5
Cutaneous epidemics

Toxic agent	Product	Contaminated ime	Ill	Deaths	Country	Year
HCH	House walls Bedcovers	1–2 months	69	6	Greece	1951
Phosdrin	Jeans	1 month	6		USA	1961
PCP	Nursery Linen	5 months	20	2	USA	1967
HCP	Talc	6 months	204	36	France	1972
Warfarin	Talc	3 months	741	177	Vietnam	1981
DEET	Insect repellent		5		USA	1989

York State Department of Health, in August 1989 [14]. Four boys aged 3–7 years and one 29-year-old man had generalised seizures temporally associated with topical use of *N,N*-diethyl-*m*-toluamide (DEET) as an insect repellent.

4. Occupational epidemics

The occurrence of collective occupational poisoning by pesticides is an habitual event in any country.

The origins of occupational exposure leading to collective poisoning are lack of safety measures in manufacture, lack of safety measures in spraying – lack of protective clothes and safe closed systems and excessive concentrations and time of exposure – and lack of safety measures in harvesting – lack of protective clothes, entry of workers not in compliance with the specific intervals and degradation products more toxic than the original product [15].

Among the high number of incidents reported we have chosen some selected examples to illustrate the different types of problems implicated.

In the Kepone outbreak in Virginia in 1974, in the manufacturing field, about 30 workers were hospitalised showing tremors in limbs, disturbance of vision, weight loss, mental changes and abnormal sperm motility [16]. In all 76 of 133 workers exposed presented some symptoms such as liver enlargement and splenomegalia. In July 1975 the plant was officially closed as a public health hazard.

The largest sprayer episodes occurred in 1976 among 7500 field workers in the Pakistan malaria control program using malathion formulations [17]. In the peak month of the epidemic it was estimated that there were about 2800 cases with 5 deaths (2 mixers and 3 spraymen). The epidemic was related to poor work practice, developed when DDT was the insecticide used for malaria control, and the contamination of malathion formulations with isomalathion.

In California in 1989 thirty-five workers became ill after harvesting for about an hour in a cauliflower field that had been sprayed 20 h earlier with organophosphate insecticides, mevinphos and oxydemeton-methyl and the carbamate, methomyl [18]. California regulations require a safety re-entry interval of at least 72 h. All crew members presented cholinergic signs and symptoms and a depression of red blood cholinesterase activities.

A very different kind of occupational exposure to pesticides has been described among office workers after pesticide application in buildings for insect infestation [19]. Standards of re-entry times after organophosphate application in California did not apply to offices or residences elsewhere, nor were there restrictions on organophosphate use during times of building occupancy.

5. Sarin poisoning

Sarin (isopropyl methyl phosphonofluoridate) was synthesized by the Germans during the Second World War as many of its related compounds. Because of high toxicity and physical properties it is one of the the most potent chemical warfare agents and has been discarded for use as an insecticide. The inhibitory activity of sarin against the cholinesterase enzymes seems to be 4000 times higher than that of parathion [20].

In 1974 the first report of human accidental exposure to sarin was published describing the clinical picture of 1 isolated and 3 clustered cases of sarin poisoning at the workplace [21].

In the last year 2 collective exposures to this gas have broken out in Japan. In June 1994 there was an incident in central Japan in which 200 people were affected and 7 died [22]. On March 20 1995 this agent was used again in a terrorist attack on the Tokyo subway killing 10 people and poisoning over 5000 [23]. The first report on clinical symptoms showed the typical muscarinic, nicotinic and central pictures with reduced consciousness levels, miosis, fasciculations, flushing, tachycardia, raised blood pressure, respiratory distress and flaccid paralysis in the severest cases. Although some authors found nicotinic-dominant responses, the treatment that followed was a combination of atropine sulphate, pralidoxime iodide and diazepam. Surprisingly, many cases

improved with the use of only atropine eyedrops. This episode constitutes a very good example of the importance of careful study of toxic outbreaks.

So there are some well-known aspects of sarin toxicity: it acts, as is expected, on the basis of structure, by blocking the activity of esterases in vitro, producing a matching clinical picture in experimental animals and in the few human exposures reported before the Tokyo incident. However, to conclude on the effects of exposure of large human populations, what appears to be essential is a careful study of this unwanted collective exposure.

The following example can stress this point: latest experimental research [24] indicates that, contrary to what has been stated before, sarin has not only a peripheral mode of action in the nervous system but also a central toxicity which, as in the case of soman, results in cellular lesions. These lesions have been found in the hippocampus, piriform cortex and thalamus, showing a significant decline in the area of CA1 and CA3 hippocampal cells as well as in the numbers of CA1 cells. These findings are similar to those described after exposure to soman. The lesions seem to increase with time, affecting broader regions 3 months after exposure. Therefore it seems imperative to look for the clinical expression of these lesions in exposed humans, mainly impairment of fixation memory, in order to confirm that this kind of toxic effect can also be expected in man.

Therefore toxic epidemic studies are useful not only to help in the prevention of those episodes but also to enlarge our knowledge of toxic effects in man, and to avoid extrapolation difficulties.

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Pesticide exposure assessment

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Abstract

Unintended, accidental, or unavoidable human exposures may result from pesticide use. Risk Characterization provides registrants, regulators, and the public a means to assess relative risks of pesticide use. Exposure Assessments are less standardized. Potential Dermal Exposure (PDE; mg/kg) is the amount of contact with the potential for dermal absorption (DA). Mixer/loader/applicator data developed using passive dosimetry and skin washing forms a Tier 1 Generic Database. If disqualifying estimates are obtained a more accurate estimate (Tier 2) may be developed from measurements of DA, clothing protection, and PDE under use conditions. Direct estimates of absorbed dose (Tier 3) require metabolic and kinetic data and biological monitoring. Harvesters and other persons who contact treated surfaces need reentry intervals to minimize acute and chronic exposure. Work tasks, dislodgeable foliar residue, and duration of exposure are the foundations for exposure-based, generic estimates of harvester PDE.

Keywords: Pesticide; Human; Risk assessment; Passive dosimetry; Biomonitoring; Risk management

1. Introduction

As a result of the widespread use of risk assessment paradigms by pesticide registrants, regulators, and the public, increased attention has been given to the development of pesticide exposure assessments. This activity follows the 1983 publication by the National Research Council of *Risk Assessment in the Federal Government: Managing the Process* [1]. The elements hazard identification, dose response assessment, exposure assessment, and risk assessment embody this extremely useful tool for Risk Management (Table 1).

Human exposures whether unintended, accidental, or unavoidable occur as a consequence of pesticide use or persistence in a variety of media including air, water, soil, plants and animals (especially foods), and on inanimate objects. Such exposures differ over many orders of magnitude and virtually none is of health or environmental significance. This contribution will consider unavoidable pesticide exposures that are associated with intentional use of chemical technologies. These exposures are for the most part occupational, and, in some circumstances, they may be useful to rank the exposures of others. Attention will be given to methods and techniques currently used to gather human exposure data and to its use in the regulatory arena.

Pesticide use occurs under specific label instructions, and under usual circumstances the formulated active ingredient is dispersed through the air. Unintended and accidental exposures result when the formulation or its active ingredient do not impact the intended target, the differ-

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Table 1
Unit processes and default assumptions of current risk assessment paradigm

Activity	Critical default assumptions
Hazard identification	Humans respond at same dose as most sensitive animal
	Risk can be assigned to a single molecule
Dose-response studies	Chronic exposures are more risky than acute exposures
	Biological processes are related to surface area
Exposure assessment	
Tier 0	Assumes uniform distribution of dose
Tier 1	No protection from clothing
	100% dermal absorption
Tier 2	Experimental dermal absorption applies under natural conditions
	Clothing and equipment permeation can be studied using passive dosimetry
	Maximum use rates and minimum re-entry intervals
	Lifetime exposures are <40 years
	Experimental conditions do not alter work practices
Tier 3	Low and high dose kinetics and metabolism are same
	Minimal genetic variability
	Compliance with study protocol

Default assumptions are used as general knowledge when specific knowledge is lacking.

ence between the two terms being subjectively dependent upon amount and the disposition of the receptor. Those types of exposures will not be mentioned further. The focus of the remainder of this presentation are the predictable, low-level, μg–mg exposures resulting from routine use of pesticide products.

2. Handlers: Mixer/loader/applicators

Persons who handle, mix, or load pesticide formulations have the highest exposure potential and at the same time the highest degree of protection from engineering controls and personal protective equipment.

These workers were the first persons monitored for pesticide exposure in the early 1950s. Griffiths et al. [2] measured parathion in the air stream, and later in 1954 Batchelor and Walker [3] used patches to intercept potential dermal exposure. The monitoring methods and techniques of those times were described in detail in 1962 by Durham and Wolfe [4]. The overall goal of early efforts was establishment of a safe workplace, rather than estimation of Potential Dermal Exposure or Absorbed Daily Dosage. As result of those efforts, engineering controls such as enclosed cabs, closed transfer systems, improved hose fittings and couplings, personal protective equipment, and formulations with low exposure potential (powders vs. granules vs. liquids) were introduced. Sub-chronic exposures of professional applicators may also require detailed description (e.g. months/year, weeks/month, days/week, h/day) to qualify certain products for particular patterns of use involving repeated exposures of pesticides with low No Observed Adverse Effect Levels (NOELs). When combined with administrative controls of hazardous work practices, the risk manager has well-proven responses available to maintain a healthful workplace.

As a result 'unabsorbed' pesticide was usually very prominent in the exposure estimate – pesticide extracted from patches worn outside or inside the outer garment and washes or rinses of the hands, V of the neck, face, back of the neck. It may be too much to expect that these data can be easily and accurately converted to ADD as required for contemporary risk assessment. Too few tests utilizing passive dosimetry coupled with biomonitoring have been performed to be able to evaluate and validate the quality of that database with respect to today's need for measures of internal dose.

Exposure estimates based upon unabsorbed dose are additionally weakened by incomplete information about the distribution of dose in

spite of substantial data indicating that different regions of the body have differing propensities to absorb chemicals [5]. The distribution issue is most elegantly presented by Fenske and collaborators who have fortified tank mixes with fluorescent tracers which enables investigators to visualize the distribution of pesticide under use conditions [6]. Similar procedures have been used in the training and certification of pesticide handlers [7]. In addition to serving as a means to evaluate exposure, the fluorescent images should serve to remind investigators of the unevenness of the distribution and its consequences in passive dosimetry.

3. Tier classification of exposure data

The National Agricultural Chemicals Association and pesticide regulators from the U.S. Environmental Protection Agency and Health and Welfare Canada combined to produce a Pesticide Handlers Exposure Database (PHED) which provides generic mixer/loader/applicator exposure data [8]. Handler exposure has been demonstrated to be formulation and application equipment dependent and independent of active ingredient per se. PHED was developed to standardize exposure estimates and to develop a statistical database from individual studies that frequently involve small numbers. The fully computerized PHED system can be used to develop quantitative measures of exposure for several subsets of data representing formulations and various use scenarios.

Patch and skin wash data are used to describe a large variety of handler exposure scenarios which comprise a Tier 1 generic database (Table 1). It is very important to recognize that the current need in risk assessment for estimates of ADD (mg/kg) is being answered by data obtained with methods used to measure external dose or potential dermal exposure.

Similar systems which model worker exposure data in England, Germany, and The Netherlands have been developed, and considerable effort is currently being expended to develop a single regulatory database for Europe. The input and output data from PHED and several European systems have been compared [9].

When the Tier 1 estimate does not qualify for the intended use, a more refined exposure model may be developed. Typically clothing permeation and dermal absorption in humans, non-human primates, swine or other laboratory animals will be considered, and the pattern of use may be refined. Personal protective equipment may be introduced as a mitigating factor. As a result the Tier 2 exposure estimate is usually at least an order of magnitude less than the Tier 1 projection.

Tier 3 represents a full experimental evaluation of worker exposure utilizing biomonitoring to establish ADD. Such studies require knowledge of route of exposure and metabolic pathways in studies with good mass balance. Use of excessive dosages in experimental animal studies may limit their usefulness in forecasting metabolic fate of an active ingredient [10,11]. Similarly, reliance upon human studies derived from accidental exposures or suicide attempts may result in poor correspondence of metabolic profiles between the workplace and literature. With knowledge of metabolic fate, stoichiometry, and clearance mechanism, investigators are in a position to conduct worker monitoring studies which yield the most sound data available concerning the magnitude of human exposure.

To test Tier 1, 2 or 3 exposure estimates for either handlers or harvesters and to evaluate their associated default assumptions, exposure estimates from passive dosimetry and estimates derived from biological monitoring should be of the same magnitude. Some of the elements used to evaluate exposure estimates from harvester data are listed in Table 2.

4. Harvesters and others contacting treated foliage

Episodes of poisoning among fieldworkers are uncommon and unfortunate evidence of excessive exposure among harvesters [12] resulting from inadequate decay of foliage residues. The protection of reentry intervals and work clothes are the primary means to minimize harvester

Table 2
Absorbed daily dosage of harvesters based upon a generic transfer factor and urine biomonitoring

Tier 1
1. Potential dermal exposure = Dislodgeable foliar residue × transfer factor × time

Tier 2
2. Clothing penetration = 10% PDE = Daily dermal exposure
3. Dermal absorption = 0.1–35%/24 h = Absorbed daily dose
4. ADD/body weight = Absorbed daily $dosage_{generic}$

Tier 3
5. UDB/body weight = Absorbed daily $dosage_{biomonitoring}$
6. Parent MW/biomarker MW × 100/urine % × 100/mol % × UB = UDB
7. Urine biomarker conc. × urine volume = Urinary biomarker

exposures. Crops such as grapes, tree fruits, strawberries, and hand-harvested crops present a special challenge due to their acute and chronic exposure potential over an extended growing season. In some cases, reentry intervals must be set based only upon the measured pesticide decay curve, e.g. dislodgeable foliar residues, and the NOEL in animals.

Earliest attempts to use foliage residue data applied an 'equilibrium model' to leaf or surface pesticide levels and skin levels. Knaak et al. [13] studied the relationship between dermal organophosphate exposure and acetylcholinesterase inhibition for determination of safe levels. In addition to being limited to anti-AChEs, this procedure for determination of safe levels has not been of general usefulness due to use of 3-day AChE inhibition and the assumption of equilibrium exposure. Since the initial concern about acute toxicity has been broadened to include developmental and reproductive toxicity as well as oncogenicity, it is imperative that reentry be exposure-based rather than driven by response.

Since there are limited means to mitigate harvester exposures, there has been considerable study of environmental data to predict worker exposure. Initial efforts have concentrated upon 'unabsorbed' pesticide on clothing and skin using methods employed in early studies with handlers. DFRs were used by Zweig et al. [14] and Nigg et al. [15] to develop a first approximation of PDEs for harvesters. They plotted exposure (μg/h) as a function of DFR ($\mu g/cm^2$) and approximated the resulting slope as an empirical transfer coefficient (TC), 5000 cm^2/h. Given DFR and time, the potential dermal exposure rate was calculated as follows:

$$\text{PDE (mg/h)} = \text{DFR } (\mu g/cm^2) \times h \times \text{TC } (cm^2/h)$$

In 1990 a series of field studies from California were published [16] to show how foliage contact and work task influenced the magnitude of the transfer coefficient or transfer factor (TF; to emphasize its empirical nature). Mechanical harvesting was associated with very little exposure (TF = 1000 cm^2/h). Harvest (gloved) of row crops involving reaching and picking such as strawberries, pole tomatoes, or lettuce yielded TFs from 1000 to 17 000 cm^2/h, and TFs for tree fruit ranged from 6000 to 54 000 cm^2/h. Although imperfect, the importance of work task must not be overlooked in developing an estimate of PDE.

Several estimates of the TF for strawberry harvesters have been made. They illustrate how the method used to assess exposure can influence this important indicator of exposure potential and reveal a lack of knowledge of the exposure process. In the mid-1980s, a transfer factor of 5000 cm^2/h was applied to DFR based upon the work of Zweig et al. [14] and Nigg et al. [15]. Thus PDE was 6.0 mg for an 8-h day of harvesting berries from plants with a DFR of 0.15 $\mu g/cm^2$. This would represent an AD of 90 μg. Using whole body dosimeters and gloved harvesters, the TF was estimated to be 250–650 cm^2/h [17]. Recent biomonitoring studies included gloved and ungloved harvesters and allowed direct calculation of AD. In this case the AD

(average two 10-h days at DFR of 0.15 $\mu g/cm^2$) was about 25 $\mu g/h$. Back-calculating by 100/15 (dermal absorption) and 100/10 (clothing permeation) yields a PDE of 294 $\mu g/day$. The PDE calculated from biomonitoring data is substantially less than that calculated from passive dosimetry suggesting that the elements of monitoring systems must be carefully evaluated and adjusted to reflect lower than expected exposure (Table 2).

Hands are most heavily exposed under most circumstances. They comprise but 4% of body surface area yet they are generally recognized as the primary source of absorbed dose based upon hand rinse studies. Recently, strawberry harvesters with or without rubber latex gloves were biomonitored for malathion exposure (Table 3). Absorbed dose was reduced about 50% during 3 days of picking to demonstrate the importance of contact transfer. The other 50% of the AD likely results from contact or air particle transfer with other body parts. Glove retained malathion and rinses were a very poor predictor of AD.

The potential role of DFR in determining PDE must not be overlooked. In the simplest case, exposure potential is reduced as a consequence of the first order decay of DFR. At the time the 1990 data were assembled [16], the TF of 390 000 cm^2/h for captan on plums was considered an outlier perhaps related to aging of the residue and an extremely low DFR (0.02 $\mu g/cm^2$). A recent biomonitoring study in strawberries (Krieger, unpublished data) treated 1-month earlier with malathion yielded significant absorption at a DFR of 0.005 $\mu g/cm^2$. The resulting $TF_{biomonitoring}$ was about one-third that obtained with 3- or 4-day-old malathion foliar residue. Similar biomonitoring data are not generally available since estimates of contact transfer are usually made as close to the reentry interval as feasible. If residues become less available with time, it may represent an additional means to lower harvester exposure.

Table 3
Observed and estimated contribution of hands of strawberry harvester to absorbed daily dose of malathion[a]

Tier	Absorbed dose (μg equiv./day)		
	Day 1	2	3
1 Generic			
100% glove rinse	212	872	946
95% glove rinse	201	828	899
2 Experimental			
50% glove rinse[b]	106	436	473
3 Biomonitoring			
Observed hand absorption (bare-hand – gloved)	27	311	93

[a] Studies sponsored by California Strawberry Commission, 1994.
[b] By biomonitoring it was determined that gloves reduced exposure about 50%.

5. Turf exposures

Considerable attention has been given to the establishment of safe reentry levels based upon measurements of DFR. Gunther et al. [18] suggested that residues were mechanically dislodged from tree surfaces during routine work activities. Tasks with greater contact with treated foliage would be associated with greater exposure. If foliage levels can be used to predict exposure, risk managers have an environmental indicator to evaluate before persons engage in substantial and prolonged contact. This general goal is carried to studies of turf and indoor residues as well.

Turf including leaf blades, thatch and roots has more complex leaf surfaces which carry lower residues than simple leaves. Cut samples have a tendency to dehydrate due to a high surface to volume ratio. DFRs tend to be low on turf compared to leaf levels on crops and tree fruit. Initial turf levels were 0.14 $\mu g/cm^2$ for chlorpyrifos (1.8 lbs a.i./acre) and <0.1 $\mu g/cm^2$ for dichlorvos (1.6 lbs a.i./acre) and decreased overnight by an order of magnitude. Formulation is also an important determinant of the magnitude and persistence of pesticides on turf [19]. Formulation effects may be pronounced in exposure studies in turf due to the use of granular and encapsulated formulations, heterogeneity of the turf matrix, vertical alignment of leaf blades, and microenvironmental conditions.

With respect to the transfer factors listed in the previous section, the transfer factor for turf is

likely to be <2000 cm^2/h and resulting exposure low.

6. Indoor exposures

A 1989 paper by Berteau et al. [20] evoked considerable regulatory activity and ultimately increased attempts to determine the extent of exposure derived from use of insecticides indoors. Priority illness cases were cited, and subsequent review indicated that the majority of cases were due to malodor based upon the time to effect, nature of response and potential for contact. Worst case dosages as high as 50 mg/kg were forecast.

In order to forecast potential indoor exposures a testing scheme featuring humans wearing whole body dosimeters performing a specified set of Jazzercize™ was developed. Physicochemical surveillance of air and surfaces provided environmental data, and volunteers easily performed a 20-min routine which maximized contact with the insecticide-treated floor. Dislodgeable residues were collected using a 25-pound roller and cotton dosimeters [21]. Highest exposure potential was associated with complete discharge foggers, and exposure estimates from a chlorpyrifos-allethrin unit were published [22]. Recent studies indicate that this system can result in an over-estimate of human exposure potential. Opportunities to monitor persons who use similar products under natural conditions – situational monitoring [23] – should help guide future testing.

7. Human studies review

There is general recognition of the importance of studying human pesticide exposures in the workplace. Chemicals in the workplace are generally taken for granted, and the idea of studying them, particularly at no effect levels, is of uncertain significance to most people. Both monitoring and experimental exposure studies ethically and legally (under some circumstances) require full and complete disclosure of purpose and methods with potential volunteers [24]. This issue is discussed in more detail in a previous publication [25]. Given that the toxicity of pesticides is usually overrated, and that exposure is considered by some to be an adverse effect, it should not be surprising that exposure studies may attract more than their share of public or workplace attention. Reviewed protocols are one small means of helping to assure some persons that human studies have a degree of institutional support.

8. Concluding comments

Substantial data have been collected during the 40 years that exposure monitoring has been performed in the workplace. The methods and techniques of investigators [2,3] have not varied substantially, and useful generic data bases are being developed to augment the regulatory process. The risk assessment process has placed a high premium on an estimated low dosage, notwithstanding the fact that many present estimates are based upon default assumptions and external (direct, environmental) monitoring.

Perhaps it is time to complement the risk assessment process with greater experience-based health surveillance, and to rely more heavily upon common medical experience and biological monitoring [26]. In such a circumstance, responsible health officials and risk managers would be guided by the risk assessment and biological monitoring utilizing standards such as the biological exposure index familiar to industrial hygienists or other published standards. Contemporary risk assessments are unquestionably more complete than any previous health-based materials.

Present pesticide exposure studies are increasingly becoming more about the chemical under study and less about the behavior and exposure of persons they seek to protect (Table 1). Continued reliance on generic exposure models including unvalidated default assumptions and controlled experimental studies will continue to magnify human pesticide exposure potential. Foundation must be established through the monitoring of the activities of real people in real

time. Substantial monitoring of persons exposed to pesticides within and outside the workplace is likely to reveal lesser levels of exposure and consequently less potential impact on human health. Risk managers, regulators, the workforce and the public need to know!

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Pesticide-metabolizing enzymes

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Abstract

Pesticides are known to function as substrates, inhibitors and inducers of drug-metabolizing enzymes, with the same compound frequently acting in more than one of these roles. Current studies of phase I metabolism of pesticides include cytochrome P450 (P450) and the flavin-containing monooxygenase (FMO), with particular reference to individual isozymes. In mouse liver, the level of FMO1 is gender dependent, FMO3 is gender specific, while FMO5 appears to be gender independent. The isozyme specificity of methylenedioxyphenyl synergists for induction of P450 in mouse liver involves P450s 1A1, 1A2 and 2B10, including a non-Ah receptor-dependent mechanism for 1A2 induction. The substrate specificity of mouse and human P450 and FMO isozymes is discussed.

Keywords: Pesticide-metabolizing enzymes; Cytochrome P450; Flavin-containing monooxygenase (FMO); Methylenedioxyphenyl compounds; Phorate

1. Introduction

Pesticides [1–5], as well as many other xenobiotics, are metabolized by many enzymes, including: the cytochrome P450-dependent monooxygenase system (P450); flavin-containing monooxygenase (FMO); prostaglandin synthetase; molybdenum hydroxylases; alcohol dehydrogenase; aldehyde dehydrogenase; esterases; and a variety of transferases, particularly the glutathione *S*-transferases. Of these P450 appears to be the most important, followed by FMO. While examples of both activation and detoxication may be found within any of these groups of enzymes, depending upon the substrate being examined, P450 is most important as an activating enzyme, producing reactive electrophiles that interact with nucleophilic substituents on biologically important macromolecules such as proteins and nucleic acids. It is also important that pesticides may serve not only as substrates for these enzymes but, particularly in the case of P450, may also serve as inhibitors and/or inducers. These multiple roles are illustrated by many of our studies on such pesticides as the methylenedioxyphenyl synergists, organophosphates, organochlorines and herbicide synergists as well as studies of these enzymes at portals of entry and sites of toxic action.

The techniques of molecular biology have been applied extensively to P450. To date over 200 genes have been characterized and the nucleotide and derived amino acid sequences compared. In a number of cases the chromosome location of the gene has been determined and in others the mechanism of gene expression has

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been investigated. A system of nomenclature based on protein sequence was proposed in 1987 and updated in 1989, 1991 and 1993 [6]. Under this system P450 genes are designated *CYP* (or *cyp* in the case of mouse genes), followed by a numbering system that distinguishes gene families, gene subfamilies and individual genes. The development of this system and the comparison of protein sequences has enabled an evolutionary tree for P450s to be developed. While some P450s may be substrate specific, those involved in xenobiotic metabolism tend to be relatively non-specific, although substrate preferences are clearly evident, even between non-specific isozymes. Furthermore, isozyme specificities are also known to exist involving both inhibitors and inducers of P450 isozymes.

FMOs (EC 1.14.13.8) are located in the endoplasmic reticulum of mammalian cells and are involved in the monooxygenation of a wide variety of xenobiotics. FMOs have a similar distribution and function to many of the isozymes of P450. Originally described as an amine oxidase [7], FMO is now known to catalyze the oxidation of many organic, and some inorganic, chemicals [8,9]. The FAD prosthetic group first reacts with NADPH and then molecular oxygen to give rise to the enzyme-bound hydroperoxyflavin responsible for the oxidation of suitable substrates. These initial reactions occur in the absence of substrate and the enzyme exists primarily in the hydroperoxyflavin form [10–12]. A consequence of this is that substrates, with few exceptions, have the same V_{max}, although K_m may vary. At least 5 different isoforms of FMO have been demonstrated, some of which appear to be associated with particular tissues or cell types [13–15].

2. Xenobiotic specificity: metabolism, inhibition and induction

2.1. Substrate specificity

P450 isozymes are responsible for many different monooxygenations and for most of these reactions there are examples of pesticide substrates. For example: epoxidation (aldrin); *N*-dealkylation (atrazine); *O*-dealkylation (chlorofenvinphos); *S*-oxidation (phorate); desulfuration (parathion). Substrates for the FMO have very diverse chemical structures, from inorganic ions to organic compounds. All, however, are soft nucleophiles, a category that includes many organic chemicals but particularly organic chemicals with a sulfur, nitrogen, phosphorus or selenium heteroatom. Although xenobiotic metabolizing isoforms of P450 appear to prefer hard nucleophiles as substrates, most, if not all, substrates for FMO are also substrates for P450. However, even when the same substrate is oxidized by both FMO and P450, in addition to rate differences, there may be different products and different stereochemistry. The amount of various isoforms of both FMO and P450 may also vary from tissue to tissue. Pesticides known to be substrates for the FMO include the organophosphates, phorate and disulfoton, the carbamates, aldicarb and methiocarb, the dithiocarbamate herbicide, sodium metham, the naturally occuring insecticide, nicotine, and the trivalent organophosphorus cotton defolient Folex [16].

2.2. Methylenedioxyphenyl (MDP compounds)

Piperonyl butoxide (PBO) and sesamex (SES) have been used as synergists with pyrethroid and carbamate pesticides, and isosafrole (ISO) and safrole (SAF) are found in many common foods of plant origin, SAF having been shown to be a liver carcinogen in rodents at high doses. MDP compounds affect multiple enzyme pathways [17,18], including the P450-dependent monooxygenase system. The effect of MDP compounds on P450s is biphasic, with an initial inhibition of activity followed by an increase above control levels [19,20]. The inhibitory effect of MDP compounds has been attributed to the formation of a stable metabolite complex between the heme iron of the P450 and the carbene species formed when water is cleaved from the hydroxylated methylene carbon of the MDP [21]. MDP exposure induces several P450 isozymes not found in detectable quantities in unexposed animals [22–25].

Several studies have been published regarding the effects of MDP compounds on mammalian liver enzymes (see [25] for references). Cook and

Hodgson [26] showed that ISO increased the level of Ah receptor in mice but did not displace receptor-bound 3-methylcholanthrene (3MC) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), both of which interact with the Ah receptor to induce P450s. Cook and Hodgson [27] also demonstrated that there was comparable induction of P450 in a congenic strain of C57 mice which lacked a functional Ah receptor and in Ah receptor-proficient C57 mice. MDP compounds have been reported to induce P450 isozymes la-2 and 2b-10 in the mouse [22]. Some investigators have reported that P450 1a-1 is also induced in mice by MDP compounds [28]. In induction studies in rats using 4-*n*-alky MDPs, the length of the alkyl side chain affected which P450 isozymes were preferentially induced, with the 6-carbon side chains favoring 2B1, the rat gene most similar to mouse 2b-10 [28]. In another study, MDP compounds with electron-donating side chains were reported to be P450 inducers, while MDP compounds with electron-withdrawing groups were not [29].

Regulation of P450 isozymes 1a-1, 1a-2, and 2b-10 by MDP compounds was studied in our laboratory by measuring levels of mRNA, protein and enzyme activity in hepatic tissue from C57BL/6 (Ah+) and DBA/2 (Ah−) mice dosed with ISO or PBO [23–25]. Increases in 1a-2 and 2b-10 were observed for ISO and PBO in both strains of mice, suggesting an Ah receptor-independent mechanism for induction of these isozymes; 1a-1 induction, however, was seen only in C57 mice and only at high doses of PBO. Dose-response studies showed maximum inducible levels for 1a-2 and 2b-10 protein, beyond which the mRNAs continued to increase while the protein levels remained constant.

Further studies of the induction of the P450 isozymes 1a-1, 1a-2 and 2b-10 were carried out [23–25] using 4 MDP compounds, SAF, ISO, PBO, and SES, and the non-MDP analog of SAF, allyl benzene (AB), in male C57BL/6N mice. P450 1a-1 was not detected in control animals, and was induced by SES and PBO, with SES inducing higher levels of 1a-1 protein than PBO. P450 1a-2 mRNA was detected in the livers of control animals and was increased by all MDP compounds (SES > PBO ≅ ISO > SAF), although SAF treatment did not increase 1a-2 protein. P450 2b-10 mRNA and protein, not detected in untreated animals, were also increased by MDP compounds (PBO > SES ≅ ISO > SAF). AB treatment did not induce detectable levels of 1a-1, 1a-2, or 2b-10, suggesting that the methylenedioxy moiety is important in induction.

Recent studies [30], using the closely related benzodioxoles 5-*t*-butyl-1,3-benzodioxole, 5-*n*-butyl-1,3-benzodioxole and 5-(3-oxobutyl)-1,3-benzodioxole, have further defined the involvement of this class of compound in the regulation of P450 isozymes, confirming that even in the Ah + C57BL/6 strain of mice, none of these compounds induced cyp1a-1. All 3 induced protein and mRNA message for cyp1a-2 while the *t*-butyl analog also induced both protein and message for cyp2b-10.

Thus all of the studies of the effects of MDP compounds on P450 in mice indicate that cyp1a-2 is induced by a non-Ah receptor-dependent mechanism. This is currently being explored.

2.3. Tridiphane

The herbicide synergist, tridiphane, (2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl) oxirane) is a postemergent herbicide used as a herbicide synergist in conjugation with atrazine, its activity being attributed to its ability to inhibit glutathione *S*-transferases. Tridiphane is also known to be a peroxisome proliferator and to induce epoxide hydrolase in rodents [31].

Tridiphane is an excellent example of a pesticide that can function both as an inhibitor and an inducer of P450 with different isozymes specificities for each activity. Tridiphane appears to selectively inhibit P450 2b-10 [32] while inducing P450 4a-1 [33].

2.4. Mirex

Previous studies in our laboratory [34] demonstrated the induction of P450 2b-10 protein and associated enzymatic activities by acute exposure to both mirex and chlordecone (Kepone). Subsequently Adams et al. (unpublished data) showed that chronic low-level dermal application

of mirex also induced P450 2b-10 in mouse liver. The enzymatic activities measured, however, suggested that in addition to 2b-10 other P450(s) were induced. We have recently demonstrated the induction of 2 P450s in addition to cyp2b-10, namely P450 1a-2 and 3a. It is of interest that both 1A2 and 3A3/4 are constitutively expressed in both human and mouse liver. Since P450 3A3/4 is known to be one of the major forms in human liver and to be involved in steroid hydroxylation we have initiated studies in conjunction with Dr G. LeBlanc to determine the effect of mirex induction on testerone hydroxylation in both male and female mice. These studies reveal differences both in the total amount of hydroxylated metabolites produced and in the relative amounts of different products within that total, with male mice being significantly more affected than female.

2.5. Human isoforms

Recently (Rose et al., unpublished data) we have been investigating the activity of human liver microsomes and expressed human isoforms toward pesticide substrates. The activity of human liver microsomes toward ethoxyresorufin, phorate and parathion was about 10% of the activity of mouse liver microsomes toward these same substrates although hydroxylation of *p*-nitrophenol was similar for both species. The human isoforms were expressed in human lymphoblastoid cell lines (Gentest Corporation, Woburn, MA) except in the case of isoforms from the 2C family that were expressed in yeast by Dr J.A. Goldstein (NIEHS). These studies revealed that P450s 1A2, 2C8, 2C9, 2C18, 2C19, 2E1 and 3A4 are all capable of metabolizing phorate to phorate sulfoxide. However, P450 2C18 is clearly the most active.

Although parathion was metabolized by human liver monooxygenases in a reaction requiring NADPH, the oxidation product paraoxon was not observed, only *p*-nitrophenol being detected. Further studies revealed that paraoxon, on incubation with human liver microsomes in the absence of NADPH, was rapidly metabolized to *p*-nitrophenol, a reaction not affected by carbon monoxide. These results strongly suggest that parathion is converted to paraoxon which is then rapidly hydrolyzed by microsomal esterases to *p*-nitrophenol.

3. Factors affecting activity

Many studies have demonstrated that the oxidation of xenobiotics, including pesticides, can be affected by both endogenous and exogenous factors. The former includes species, strain, age, gender, and hormonal status while the latter includes such factors as stress and diet. Most of these studies have been carried out on reactions mediated by the P450-dependent monooxygenase system and, in the case of pesticides, have seldom been carried out on individual isoforms either at the level of protein or mRNA message. Virtually nothing is known of the role of exogenous or endogenous factors on the metabolism of pesticides by FMO.

Recently [35] we have examined the role of gender in the expression of FMO isoforms in mouse liver. While it has long been known that the FMO activity toward several substrates was higher in the liver of female than that of male mice, these studies were carried out at the level of substrate oxidation and before it was known that several different isoforms of FMO exist in mammals. Hepatic FMO activity of microsomes from adult CD-1, Swiss-Webster, C57BL/6 and DBA/2 mice was found in all cases to be significantly higher in females than in males. Based on protein and mRNA levels in CD-1 mice, it was shown that the isoforms responsible for this difference were FMO1 and FMO3, FMO5 being the same in the livers of either gender. FMO1 was 2–3 times higher in females than in males while FMO3, expressed at levels similar to those of FMO1 in females, was not detected in males. There was close correspondence between protein levels and mRNA levels in each case. Thus in mouse liver there is a gender-independent isoform, a gender-dependent isoform and a gender-specific isoform. Neither FMO2 nor FMO4 were expressed in the liver of mice of either gender. This effect, while dramatic, is tissue dependent. FMO1, FMO3 and

FMO5 are expressed at similar levels in the lung and kidney of CD-1 mice of either gender.

4. Alternate pathways

4.1. General approach

Since P450 and FMO have many substrates in common but, at the same time, these substrates may have different products with different toxic potencies, it is important to know the relative contribution of the 2 pathways to the metabolism of a particular substrate. Furthermore, in contrast to FMO isozymes, xenobiotic-metabolizing isozymes of P450 are often relatively easily induced, thus making the relative contributions variable with the conditions of exposure. Although it is said that the FMO prefers soft nucleophiles as substrates and P450 hard nucleophiles, with the exception of compounds oxidized at carbon atoms, this applies only to the relative ability of compounds to serve as substrates for one or the other, since it is difficult to find more than a very small number of FMO substrates that are not also substrates for one or more P450 isozymes.

Such substrates may have complex oxidation patterns and show regioselectivity in the sites attacked, they may yield different products, or different isomers of the same product. A number of methods are available for determining the relative contributions of FMO and P450, including extrapolation from the properties of purified enzymes (or from isozymes cloned and expressed in expression systems), the use of product specific substrates, the use of enzyme specific substrates or the manipulation of microsomes in which both enzymes are found. This latter technique, using selective heat treatment to inactivate FMO or an antibody to the NADPH-P450 reductase to inactivate P450 has proven most useful in our hands, particularly in the case of hepatic enzymes.

4.2. Phorate

The insecticide, phorate, undergoes a complex series of oxidations (Fig. 1). The products are generally more toxic than phorate and the reaction sequence is, therefore, an activation sequence. This substrate has continued to prove useful in examining the relative importance of FMO and P450.

FMO forms only phorate sulfoxide while P450 yields additional products. The sulfoxidation reaction is stereospecific with FMO producing the (−)-sulfoxide and several P450 isozymes the (+)-sulfoxide. While both sulfoxide isomers are substrates for all P450 isozymes tested, the (+)-sulfoxide is always preferred to the (−)-sulfoxide [36]. The relative contribution of FMO to sulfoxide formation is higher in female than in male mice in agreement with the studies of gender effects described above. Although overall sulfoxide formation is higher in the liver than in any extra-hepatic tissue, the relative contribution of the FMO is higher in lung, kidney and skin, being as high as 90% of the total in renal

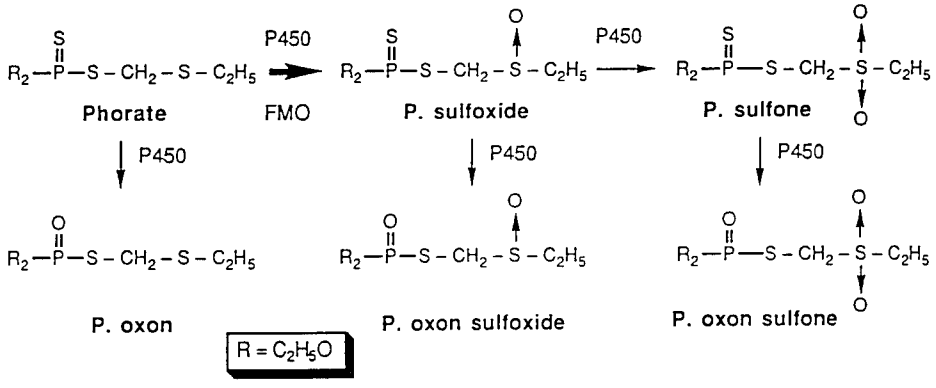


Fig. 1. Metabolism of the insecticide, phorate, by cytochrome P450 and the flavin-containing monooxygenase.

microsomes from female mice. Furthermore, the contribution of FMO relative to P450 is increased following treatment, in vivo, with P450 inhibitors such as PBO, or decreased following treatment, in vivo, with P450 inducers such as phenobarbital [37,38].

5. Portals of entry and target tissues

Alternate pathways and the relative importance of different enzyme systems in non-hepatic tissues is of considerable importance. In the case of portals of entry, not only is this the first line of oxidative attack but in both pulmonary and dermal entry, first pass through the liver is avoided. In the case of target tissues it is probable that events taking place close to the site of action, while quantitatively lower than similar events in the liver may, by proximity, be more important.

5.1. Metabolism in target tissues and portals of entry

The general methods for examination of the importance of different oxidative pathways and the metabolism of the insecticide phorate have been discussed [2,4,5] along with the expression of the FMO in target tissues and portals of entry. We have also studied additional pesticide substrates in mouse skin [39]. In these studies the P450 content, the cytochrome *c* reductase activity, the metabolism of a variety of P450 pesticide substrates, and the presence and role of FMO in xenobiotic metabolism were studied in skin microsomes and compared to those of liver. The P450 content of skin as determined by CO-dithionite-reduced minus CO-oxidized spectra was approximately 6.8% of the liver P450 content. By comparison, cytochrome *c* reductase activity in skin microsomes was high, being equivalent to approximately one-third of the liver microsomal enzyme activity. Skin microsomes metabolized several known P450 substrates and, depending upon the substrated used, the specific activity ranged from 2.5 to 13.4% of the corresponding rates seen in liver microsomes. Skin microsomes exhibited the highest enzymatic activity with benzo[*a*]pyrene and ethoxyresorufin, moderate activity with parathion and aldrin and low activity with benzphetamine and ethoxycoumarin. Skin microsomes also metabolized the triazine herbicides atrazine, simazine, and terbutryn, with the activity being 2–5% of the liver microsomal activity. FMO activity in skin microsomes with thiobenzamide and methimazole as substrates ranged from 10 to 20% of the liver FMO activity. Immunohistochemical studies using antibodies to mouse liver FMO showed localization primarily in the epidermis. Additional studies using pig skin showed a similar distribution pattern. Antibodies developed to mouse liver FMO and the constitutive liver P450 isozyme, 1A2, showed cross-reactivity on Western blots with proteins in skin microsomes that appeared identical to the cross-reacting proteins present in liver microsomes. The relative contribution of P450 and FMO in mouse skin to the sulfoxidation of phorate was investigated and compared to that of liver microsomes. Several procedures were employed to selectively inhibit either P450 or FMO so that the role of each monooxygenase system in the absence of the other system could be determined. As in the lung and kidney FMO in the skin proved to be relatively more important than P450 for the sulfoxidation of phorate. In liver microsomes, P450 was responsible for 68–85% of the phorate sulfoxidation activity. In contrast, in skin microsomes 66–69% of the phorate sulfoxidation activity was due to FMO, while P450 was responsible for the remainder of the activity. Thus, although the overall phorate sulfoxidation rate in mouse skin microsomes was only 3–4% of the rate seen in liver, FMO appears to assume a greater relative role to P450 in the metabolic processes in skin. We have also observed, by immunocytochemical methods, the distribution of FMO in the skin. Its distribution was primarily in the epidermis.

Studies in the laboratory of Dr Mary Beth Genter (pers. commun.) utilizing immunocytochemical methods have revealed that at least 2 isozymes of the FMO are present in the olfactory epithelium. FMO3 is broadly distributed while FMO 1 has a more restricted distribution.

5.2. Thioridazine

Many drugs, including antipsychotics, monoamine oxidase inhibitors and antihistamines are substrates for the FMO. The antipsychotic drug, thioridazine (TDZ), is an excellent substrate for examining the relative importance of different oxidative pathways since it is oxidized at multiple sites by both FMO and P450 (Fig. 2).

Based primarily on examination of urinary and serum metabolite profiles, *S*-oxidation appears to be the predominant route of metabolism in humans, producing the 2-sulfoxide, the 2-sulfone and the 5-sulfoxide. The 2-sulfoxide and the 2-sulfone are known to have greater antipsychotic activity than the parent compound, while the ring sulfoxides may be responsible for the cardiotoxic side effects sometimes seen with TDZ (see [4,5] for references).

Metabolism by hepatic microsomes from the mouse yielded primarily the 2-sulfoxide of TDZ with significant amounts of the 5-sulfoxide, the *N*-oxide, the *N*-demethyl derivative and the 2-sulfoxide-*N*-oxide [40]. Heat treatment of microsomes to selectively destroy the FMO or treatment with an antibody to the NADPH-P450 reductase to inhibit P450 isozymes revealed that the *N*-oxide was the principal metabolite derived from the FMO, while the 2-sulfoxide and the other products were derived primarily from one or more isozymes of P450.

Studies using FMO purified from mouse liver have shown the *N*-oxide of TDZ to be the principal and, perhaps the only, product of TDZ metabolism by this enzyme. Similar experiments using P450 2D6 reveal that the 2-sulfoxide of TDZ (mesoridazine) is the principal, but not the only, metabolite of TDZ and this P450 isozyme. Similar experiments have been carried out with P450 isozymes 2E1 and 2B1.

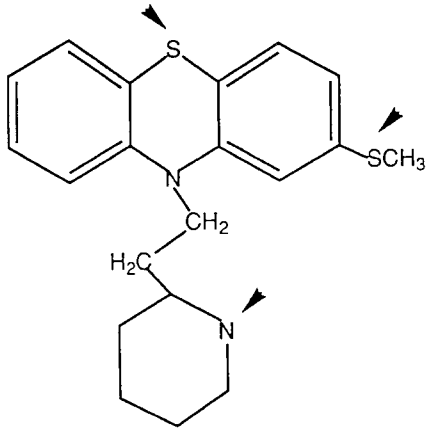


Fig. 2. Sites for oxidative attack on thioridazine.

Since TDZ and several of its metabolites show similar biological activity, the possible occurrence and role of FMO in the nervous system appears to be important. Previous studies indicating the possible occurrence of the FMO in rat corpus striatum and whole brain microsomes could not be duplicated. Our results, using microsomes prepared from mouse brain, of substrate level oxidations and Western blotting with an antibody, a form of FMO purified from mouse liver, while not negative, were equivocal. In order to determine the presence of FMO mRNA in rabbit brain we have recently (Blake et al., unpublished data), in conjunction with Dr. R.M. Philpot, utilized polymerase chain reaction (PCR) techniques to demonstrate FMO in the nervous system of the rabbit. Recently, 5 forms of MFO [1–5]) have been identified in rabbit hepatic and extrahepatic tissues [15], most tissues expressing more than one form. PCR amplification of cDNA was performed using primers specific for each of the 5 forms of FMO found in rabbit tissues. The data suggest that 1 form, apparently form 4, is expressed in rabbit brain. This FMO has recently been cloned and sequenced from a human liver cDNA library [41].

The substrate specificity of FMO4 has not been determined. If it is a metabolically active protein, the inability to detect its presence in brain is probably due to its localization in certain brain regions or cell types. Studies are in progress to confirm the presence of 1E1 message in brain and to utilize immunocytochemical and in situ hybridization techniques to localize the isozyme in the brain.

6. Conclusions

The interactions of pesticides with P450, FMO and other Phase I and Phase II enzymes have

been studied for some time. However, isozyme specificity for metabolism, induction and inhibition is still little understood. If toxicology is to serve the public interest by carrying out the kind of studies that protect public health, but at the same time allow efficient vector control and the production of food and fiber, a more holistic approach will be necessary. It is not sufficient to know whether or not an agricultural chemical is a substrate for microsomal oxidation. A number of other questions must be addressed: what are the specific enzymes and isoforms involved? is the reaction an activation or a detoxication reaction? does induction and/or inhibition occur? what is the relationship to other Phase I and to Phase II enzymes? how are these relationships changed by other xenobiotics? what interactions occur at portals of entry and sites of toxic action? Only with this type of fundamental information can applied problems in the field be addressed.

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Molecular biology of insecticide resistance

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Abstract

The widespread use of insecticides has amounted to a large scale 'experiment' in natural selection of insects by chemicals of toxicological importance to humans. Specific examples in which the molecular basis of insecticide resistance has been studied in detail are presented here. The biochemical/physiological mechanisms of resistance can be categorized as target site insensitivity, increased metabolic detoxification and sequestration or lowered availability of the toxicant. These are achieved at the molecular level by: point mutations in the ion channel portion of a GABA receptor subunit (cyclodiene insecticides); point mutations in the vicinity of the acetylcholinesterase (AChE) active site (organophosphorus and carbamate insecticide resistance); amplification of esterase genes (organophosphorus and carbamate insecticides); mutations linked genetically to a sodium channel gene (DDT and pyrethroid insecticides); and yet uncharacterized mutations leading to the up-regulation of detoxification enzymes, such as cytochrome P450 and glutathione *S*-transferases (many classes of insecticides). In several cases, the selection of a precisely homologous mutation has been observed in different insect species.

Keywords: Pesticides; GABA gated Cl^--channel; Acetylcholinesterase; Na^+-channel; Cytochrome P450; Glutathione *S*-transferases; Esterases

1. Introduction

Perhaps the most serious consequence of widespread, indiscriminate use of insecticides is the development of insecticide resistance. More than 500 species of insects and mites are reported to have developed resistance to one or more classes of insecticides [1]. A number of agricultural pests and disease vectors are so resistant in some areas of the world that chemical control has become extremely difficult. The list of effective insecticides for the control of crop pests and disease vectors is rapidly shrinking. Meanwhile, fewer new insecticides are being introduced to the market, largely because of the high costs associated with research, development and registration, and the prognosis of a limited effective lifespan of the new insecticide. The impact of insecticide resistance in toxicology is 2-fold: (1) the insecticide 'treadmill' has lead to the introduction of a huge arsenal of sometimes highly toxic chemicals in the environment, and several have become problem pollutants over the last 50 years; (2) the immediate reaction to insecticide resistance by pest control practitioners has traditionally been an increase in the dosage, thus compounding the problems of resistance and environmental contamination.

The study of insecticide resistance at the molecular level has uncovered a number of ways in which animals can survive in toxic environments. The nature of the mutational events that

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lead to resistance in insects should not be expected to be intrinsically different from those that are responsible for the genetic variation of human responses to xenobiotics. Indeed, insects and vertebrates share many of the favorite targets for 'insecticidal' action and they share many of the metabolic detoxification systems. Selectivity of insecticides is achieved either when insects and vertebrates do not share a target (e.g. the selectivity of juvenile hormone agonists) or when they follow different metabolic pathways (e.g. the low vertebrate toxicity of malathion).

Resistance marks a genetic change in response to selection. Individuals carrying genetic traits for coping with the chemically hostile environment survive and reproduce, thereby passing on these traits to their progeny. Continued selection pressure exerted by the insecticide rapidly increases the frequency of the genetic trait (resistance) in the population. I present here a brief overview of molecular mechanisms (types of mutations) that lead to insecticide resistance.

2. Physiological/biochemical and molecular mechanisms of resistance

Genetic and biochemical studies have contributed immensely to our understanding of resistance. There are 2 ways in which organisms can become resistant to xenobiotics such as pesticides: either by modifying the effective dose of the pesticide available at the target site or by modifying the target site itself. Thus, classically recognized mechanisms of resistance such as behavioral resistance, reduced penetration or absorption, sequestration and detoxification all contribute to decrease the dose of the pesticide, whereas a decreased target site sensitivity or a modification of target site number will contribute to render a dose of pesticide ineffective. In-depth discussions of these mechanisms are given in Roush and Tabashnik [2]. Molecular mechanisms of resistance are classified according to the type of genetic change selected by the insecticide pressure: point mutations in structural genes, or modifications in the number or activity of genes.

3. Point mutations in structural genes: cyclodiene resistance and acetylcholinesterase insensitivity

Nucleotide deletions, additions, or substitutions in the DNA may alter the amino acid sequence of a protein. Point mutations in the target sites of several herbicides and fungicides are known to confer pesticide resistance. Two such examples are also known in insects, and illustrate the power of *Drosophila* as a model system for insecticide resistance in general [3].

3.1. Point mutation in the Rdl gene of Drosophila [4]

Elucidation of the molecular mechanisms of cyclodiene insecticide resistance in *Drosophila* followed a logical, ab initio approach that led to the cloning of the first invertebrate GABA receptor subunit. Field-collected populations of *Drosophila* were screened for dieldrin resistance. A homozygous strain with 4000 × resistance was isolated, and the resistance gene (*Rdl*) mapped genetically at 26 cM on the left arm of chromosome III. Deficiency mapping and irradiation-induced rearrangements allowed a more precise localization to the cytological region 66F. The resistance gene was then uncovered by *P* element-mediated transformation of flies with a cosmid clone obtained from a chromosomal walk in that region. The cosmid clone contained a susceptible copy of the *Rdl* gene, thus 'rescuing' susceptibility in resistant flies. Screening of a cDNA library with a fragment of this cosmid clone yielded a clone encoding a GABA receptor subunit. This confirmed earlier pharmacological evidence that had pointed to the picrotoxinin binding site on $GABA_A$ receptors as the likely neuronal target of cyclodiene insecticides. Expression of the cDNA in *Xenopus* oocytes demonstrated a functional, homo-oligomeric GABA-gated chloride channel.

Sequencing of *Rdl* in resistant flies revealed a single common point mutation of Ala^{302} to Ser. This mutation is located in the second membrane spanning region of the channel subunit, lining the

chloride ion pore. Site-directed mutagenesis of the susceptible cDNA and expression in *Xenopus* oocytes showed that this mutation leads to channels insensitive to block by dieldrin.

Armed with a molecular diagnostic, a number of dieldrin-resistant and susceptible strains were screened. The Ala^{302} to Ser mutation was absent in 122 susceptible strains, and present in 58 resistant strains of *Drosophila melanogaster* from around the world. The only observed fitness cost of this mutation is a temperature-sensitive phenotype, i.e. temporary paralysis at 38°C. Most remarkably perhaps, was the subsequent demonstration that an identical mutation is found in cyclodiene-resistant strains of a number of other insect species. In 2 species, the homologous Ala residue is mutated to Gly instead of Ser (Table 1).

The molecular diagnostic for target site resistance to cyclodiene insecticides also allowed an accurate determination of resistance frequency in a relatively small samples of flies collected in the field. Resistance frequency was shown to be as high as 1%, even though wild populations of *Drosophila* in the US have apparently not been under cyclodiene selection pressure for many years [4].

3.2. Point mutations in the Ace gene of Drosophila

Reduced sensitivity of acetylcholinesterase (AChE) is a well-characterized biochemical mechanism of insecticide resistance. Higher K_m and lower k_i values for organophosphorus and carbamate insecticides in resistant strains indicated structural alterations in the active site of the enzyme [5]. In turn, these alterations in protein structure may be attributed to changes in the amino acid sequence of the target protein.

The structural gene, called *Ace*, coding for the AChE specifically expressed in the central nervous system of *Drosophila melanogaster* has been cloned, sequenced and extensively characterized. Because *Ace* is a single locus that was known a priori to be involved in resistance, a directed search for point mutations was justified. Comparisons of the coding sequences of the *Ace* gene of susceptible and resistant *Drosophila* strains revealed 5 point mutations at 4 positions in the resistant strains [6]. In one strain, mutations at all 4 positions are present in the same gene (Table 2). These point mutations were introduced in a minigene construct devoid of the carboxy terminus coding sequence. Thus expression of a soluble protein in *Xenopus* oocytes

Table 1
Conserved M2 region of the *Rdl* GABA receptor subunit in various insect species

Ala Arg Val **Ala** Leu Gly Val Thr Thr		
Residue at the Ala^{302} position	S-strain	R-strain
Drosophila melanogaster	Ala (122 strains)	Ser (58 strains)
Drosophila simulans (2 alleles found)	Ala	Ser/Gly
Aedes aegypti (mosquito)	Ala	Gly
Periplaneta americana (cockroach)	Ala	Ser
Musca domestica (house fly)	Ala	Ser
Tribolium castaneum (flour beetle)	Ala	Ser
Hypothenemus hampei (coffee berry borer)	Ala	Ser
Bemisia tabaci (whitefly)	Ala	Ser

Adapted from ffrench-Constant [4].

Table 2
Point mutations in AChE insensitive to organophosphates/carbamates

	Phe^{115}	**Ile**^{199}		**Gly**^{303}	**Phe**^{368}	
Drosophila S						
R Saltillo	Ser	Val	–	Ala	Tyr	–
R Bygdea	–	Val	–	Ala	–	–
R Pierrefeu	–	Thr	–	Ala	–	–
R MH19	–	–	–	–	Tyr	–
House fly S						
			Val^{180}	**Gly**^{262}	**Phe**^{327}	**Gly**^{365}
R 77M	–	–	Leu	Ala	Tyr	–
R CH2	–	–	–	Ala	Tyr	–
R 49R	–	–	–	–	–	Ala

S, susceptible strain; R, resistant strain.
Val^{180}, Gly^{262}, Phe^{327} and Gly^{365} in the house fly correspond to Val^{220}, Gly^{303}, Phe^{368} and Gly^{406} in *Drosophila*. Gly^{365} is adjacent to the Glu residue of the AChE catalytic triad.
Adapted from Mutero et al. [6]; Williamson [7].

allowed a functional characterization of these mutations, either alone, or in some of the combinations found in resistant strains collected from the field. The results clearly indicated that multiple mutations caused higher levels of insensitivity to organophosphates and carbamates [6].

Multiple mutations in the same gene may result either from the accumulation of mutations, or from the recombination between genes carrying single mutations. This interesting problem has not been clearly resolved, but there are indications favoring the second possibility. Clearly, this would have important implications in resistance management, particularly with regard to migration, population size and fitness of the individual mutants in the absence of insecticide selection.

Because the 3-dimensional structure of a model AChE, the electric organ enzyme from *Torpedo californica*, has been determined by X-ray analysis, alignment of the *Drosophila* sequence has allowed a discussion of the possible influence of these mutations on substrate or inhibitor binding in the active site gorge of AChE.

The enzyme has also been sequenced in other insect species, and recently the mutations conferring resistance in the house fly AChE have been determined [7]. Mutations at 4 sites were observed, 2 of which at residues homologous to those determined in *Drosophila* (Table 2).

The evidence gathered to date on *Rdl* and *Ace* point mutations allows 2 interim conclusions. First, that *Drosophila* is a powerful and predictive model for major insect pest species. Second, that the 'choice' of point mutations that lead to a modified sensitivity to xenobiotics upon selection is probably most limited in target molecules that are most conserved across taxonomical boundaries. This is also a conclusion that emerges from studies on herbicide resistance in photosystem II.

4. Gene amplification: esterases and resistance to organophosphorus and carbamate insecticides

DNA amplification events create additional copies of chromosomal sequences (including functional genes) which can survive in either intra- or extrachromosomal forms. This phenomenon was first extensively documented in tumor cells and permanent cell lines, for instance methotrexate resistance resulting from the amplification of the target dihydrofolate reductase [8]. Initially regarded as abnormal events occurring in tissue cultures, DNA amplification is now amply documented in whole organisms where

expression of the gene product must be faster than can be achieved by transcription from only a single copy of the gene [8]. At this point, it must be emphasized that DNA amplification per se does not bring about resistance. The amplified gene must be transcribed and translated (over-expressed) into functional protein products for expression of the resistance trait.

Devonshire and Sawicki, working with organophosphorus-resistant populations of the aphid *Myzus persicae*, first invoked a simple model of 'a succession of tandem duplications of the structural gene' for an insecticide-associated esterase (esterase E4). In a series of progressively more resistant clones, progressive elevation of titers of E4 correlated with the degree of observed resistance. Esterase E4 (and the closely related FE4) confer broad resistance to insecticidal esters [9]. In vitro translation of poly(A)$^+$RNA from 3 clones showed elevated levels of E4- or FE4-encoding mRNAs in the resistant clones. Direct evidence for an up to 64-fold amplification of the E4 esterase in *M. persicae* was obtained when a cDNA clone was used to probe the genomes of susceptible and various resistant aphid clones [9]. Reversion of resistance without loss of the amplified sequences involves in a still obscure way the DNA methylation status of the esterase genes.

Evidence for DNA amplification of esterase genes was also obtained in a number of insecticide-resistant strains of the *Culex pipiens/quinquefasciatus* complex. Resistant mosquitoes from around the world have been characterized over the last few years, revealing amplification of several closely related esterases (Table 3). Although migration was invoked as a major cause of the worldwide distribution of amplified esterase B2 [10], it is equally likely that multiple amplification events have taken place.

A molecular analysis of the amplification unit (or amplicon) of esterases in aphids and mosquitoes revealed that amplification of genomic sequences extends far beyond the esterase gene itself [9]. The B1 esterase of mosquitoes is a 2.8 kb gene found 250 times as an amplicon of approximately 30 kb, with a core of 25 kb. The E4 esterase of aphids is a 4.3 kb gene, amplified up to 64-fold as an amplicon of about 25 kb. This is consistent with observations on other xenobiotic-related amplification events in eukaryotes [8].

Because the amplified esterase can account for a very significant percentage of the total protein of the insect (up to 3% in most resistant aphids), is the amplified esterase merely a sponge that sequesters the insecticide? In other words, does resistance result from hydrolysis or sequestration of the insecticide? The kinetic constants (affinity, bimolecular rate of formation of the acylated enzyme, hydrolysis/deacylation rate) for each esterase and each substrate need to be measured. A comparison of the aphid E4 and the mosquito A2/B2 esterases [12] indicated that hydrolysis was the major role of the aphid E4 esterase, whereas the mosquito A2/B2 esterases play a predominant sequestration role (Table 4).

5. Regulatory changes in gene expression

DNA amplification is a mechanism whereby overabundance of a gene product is achieved by a multiplication of the gene itself, but there are molecular mechanisms responsible for increased

Table 3
Amplified esterases in *Culex pipiens/quinquefasciatus* mosquitoes

Origin	Esterase	Copies	Esterase levels	Chlorpyrifos resistance
North America	B1	250×	500×	800×
Africa, Asia, N. America	A2 B2	60×	–	–
France	A1	–	70×	100×
France	A4 B4	25×	50×	6.6×
Cyprus	B5	250×	500×	95×

Adapted from [9–11].

Table 4
Esterase amplification in insects: sequestration or metabolism?

	pmol/insect (% total protein)	Paraoxon	
		Sequestered (ng)	Hydrolysis/h (%)
Mosquito A2/B2	7.7	2.1	5.6
Culex quinquefasciatus	(0.4)		
Aphid E4	10	2.5	33.2
Myzus persicae	(3)		

Adapted from Karunaratne et al. [12].

expression of non-amplified structural gene sequences. Conversely, gene expression can be decreased. Various types of mutations can lead to changes in gene expression and these can occur in *cis* (for instance disruption or deletion of an upstream regulatory element of the gene, whether this element is enhancing or repressing gene expression) or in *trans* (for instance disruption of a gene coding for a protein that binds to the above-mentioned *cis* elements). It is clear that evidence for regulatory changes in gene expression are more difficult to obtain and to describe accurately in molecular terms.

Regulatory changes appear to be involved in cases of metabolic resistance, specifically in those instances where glutathione *S*-transferases (including DDT-dehydrochlorinase) and cytochrome P450 monooxygenases play a role in insecticide metabolism. Since both groups of enzymes are multigene families comprising many genes that have evolved by repeated duplication/divergence events, there may be significant redundancy in the catalytic function of the gene products. This in turn may allow mutations affecting gene expression of one or more of these genes to arise and to be selected by insecticide pressure.

Overexpression of glutathione *S*-transferases (GST) has been documented in resistant strains of several insect species. In the yellow fever mosquito, *Aedes aegypti*, overexpression of GST-2 is controlled by a *trans*-acting regulatory locus [13].

The P450 gene *CYP6A1* cloned from a resistant strain of the house fly is overexpressed in a number of strains. Gene amplification as a possible mechanism for high constitutive expression in the resistant strain was ruled out by dilution dot blot analysis of equal amounts of genomic DNA from both strains. Genetic crossing experiments mapped high constitutive expression of *CYP6A1* and insecticide resistance to chromosome II. The structural gene for *CYP6A1* was mapped to chromosome V, however. The results suggested that the gene product of the resistance gene on chromosome II is a diffusible factor which differentially regulates transcription of structural genes in susceptible and resistant strains [14]. The exact nature of the difference, i.e. the resistance mutation has not yet been determined.

The regulatory factor has a pleiotropic activity and must regulate P450 genes other than *CYP6A1*. This conclusion is based on the observation that metabolism of diazinon, increased in resistant strains, is not catalyzed by CYP6A1. Instead, CYP6A1 expressed in *E. coli*, purified and reconstituted with house fly NADPH-cytochrome P450 reductase, catalyzes the epoxidation of aldrin, heptachlor and of some terpenoids [14]. Whether changes in gene expression are involved in other cases of metabolic resistance remains to be established. Preliminary evidence for 2 other P450 genes, the *CYP6D1* gene in permethrin-resistant house flies and the *Cyp6a2* gene in DDT-resistant *Drosophila* also implicates up-regulation in *trans*. A regulatory gene mutation causing metabolic resistance to insecticides has been predicted on genetic and biochemical grounds by Plapp [15] and the characterization of this regulatory gene should prove to be rewarding.

6. Linkage of DDT and pyrethroid insecticide resistance (knock-down resistance) to a Na^+ channel locus

Knock-down resistance (*kdr*) has long been associated with the pharmacology of neuronal voltage-gated Na^+-channels. Mutations in one *Drosophila* Na^+-channel gene, *para*, were shown to have a weak resistant phenotype [16]. Recently, molecular probes for genes homologous to *para* have been obtained in the tobacco budworm, *Heliothis virescens* [17], in the house fly, and in the German cockroach, *Blatella germanica*. These probes were used to demonstrate genetic linkage between a polymorphism in the *para* homolog of these species and permethrin (*Heliothis*) or DDT resistance. Linkage was very tight in the house fly and the cockroach, and less so in the tobacco budworm [17], possibly because metabolic resistance factors in addition to *kdr* may have been present in those insects. Thus, *kdr* mutants may be structural mutants of Na^+ channel proteins. However, other mutations in *Drosophila* also affect *para* indirectly [16], and cause some degree of resistance to pyrethroids. For instance, a reduced number of nerve membrane Na^+ channels may be involved in DDT and pyrethroid insecticide resistance as well.

7. Conclusion

Great progress has been made in the last 5 years towards the elucidation of molecular mechanisms of insecticide resistance. But we have only a glimpse of the variety of mechanisms by which resistance is achieved. Work on the *Met* (methoprene tolerance) mutation in *Drosophila* which confers 100-fold resistance to methoprene, a juvenile hormone analog, should soon yield exciting new insights into the mode of action of juvenile hormone. Resistance to the insecticidal toxins of *Bacillus thuringiensis* will need to be understood as transgenic crop plants expressing these toxins reach the farm.

The demonstration of P450 gene amplification in humans (*CYP2D6* in 2 Swedish families) led Meyer [18] to pose the question whether drug-metabolizing enzymes are preferred targets for amplification in situations of xenobiotic or toxic exposure. The examples of insecticide resistance described above show that in such extreme situations eukaryotes can respond in very diverse ways, depending on the target and the chemical. This exploitation of genetic variation by insect pests will continue. We are challenged to integrate information on molecular mechanisms of resistance with population genetics with the aim of devising resistance management strategies.

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The brevity of this review has forced me to highlight and cite only some of the very nice research conducted on this subject. I acknowledge the fine contributions of *all* authors working on the molecular biology of insecticide resistance.

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Receptors as tools for understanding the toxicity of retinoids[1]

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Abstract

Retinoids are derivatives of vitamin A that have numerous biologic activities including induction of epithelial differentiation, pattern formation in embryos, and maintenance of spermatogenesis. Retinoids are used to treat various dermatologic maladies and specific forms of cancer but their use is limited by toxic liabilities: most notably teratogenesis. Retinoids interact with 2 families of receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The RARs and RXRs bind and transactivate distinct response elements of numerous genes. This multiplicity of receptors and gene products provides us with multiple targets for developing novel receptor-selective agonists. We are exploiting our knowledge of ligand receptor interactions to design better, more selective drugs and to understand the toxicity of retinoids and their metabolic products.

Keywords: Retinoid; Receptors; Gene expression; Teratogenicity

Recent discoveries on the molecular mechanisms of retinoid action provide a means for understanding the toxicity of retinoids.

Retinoids are now thought to produce their biologic effects by interacting with specific nuclear receptors for retinoic acid (RA) (reviewed in [1,2]). These receptors are members of the steroid/thyroid superfamily of receptors and as such are ligand-dependent transcription factors (reviewed in [3,4]). As the first step in the pathway of retinoid activity, the receptors are key molecular targets through which we can gain insight into the mechanism of action (and toxicity) of retinoids. Two families of receptors, the retinoic acid receptors (RARs) [1,2] and the retinoid X receptors (RXRs) [5–7], define gene pathways through the activation (or repression) of specific DNA sequences, called response elements, in the untranslated regions of retinoid-responsive genes [2]. Some response elements are specific for RXR [8,9], while other response elements have been shown to interact with RAR-RXR heterodimers [7,10]. Each of the receptor families, RAR and RXR, is further divided into 3 receptor types α, β, or γ [1,2]. These receptor types may themselves preferentially activate specific response elements [11]. We and others have recently shown that there are distinct ligand

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specificities for each of the receptor families, RAR and RXR. The RXR family of receptors binds 9-*cis* RA [12,13], while the RARs can bind all-*trans* RA and 9-*cis* RA [14]. Thus, there are multiple retinoid receptors and multiple ligand pathways. The multiplicity of receptors, and ligands combined with multiplicity of response elements provide the combinatorial possibilities required to explain the pleiotropic effects of retinoids in normal function and in toxicity (reviewed in [2]).

The role of receptors in retinoid-induced toxicity is generally well accepted and there is both indirect and direct evidence to support the concept at the present time. Indirect evidence comes from a correlation between retinoid binding and activation of the RARs in vitro and the induction of pharmacologic and toxicologic activity in whole cells or in vivo [15,16]. In general, compounds that bind to the RARs weakly are less toxic and those that bind with high affinity are more toxic. (Data are not yet available for correlating toxicity with the binding of ligands to the RXRs.) However, the biologic activity of some retinoids does not correlate with in vitro binding since these compounds exhibit little or no direct binding to the receptors yet show marked biological activity or toxicity. One example of such a compound is 13-*cis* RA, which is highly active as a therapeutic agent in acne and some forms of cancer but does not bind directly to the RARs [17]. The activity of 13-*cis* RA may be the result of its binding to some other receptor or biotransformation to an isomer of RA that binds and activates one of the known families of receptors. A technique known as nuclear receptor-dependent ligand trapping was used to demonstrate biotransformation and receptor binding in whole cells. COS-1 cells overexpressing one of the RARs were incubated with [^{3}H]13-*cis* RA for 4 h. The cells were harvested, the nuclei were isolated and lysed. The identity of the radiolabel bound to the receptors in the nuclear fractions was determined by HPLC analysis. Remarkably, little or no 13-*cis* RA was detectable in the nucleosol fraction. Only all-*trans* RA was trapped by the RARs despite that fact that the media contained greater than 95% [^{3}H]13-*cis* RA [12]. The biotransformation of retinoids to more active forms might explain some of the lack of correlation of toxicity and RA binding reported previously for some retinoids [15].

Conversely, retinoids that bind directly to receptors and that are resistant to metabolic change might be more active than their binding affinities would suggest. For example, Ro 13-7410 binds to the RARs with affinities slightly less than those for RA, but for some toxicologic endpoints Ro 13-7410 is 10–1000-fold more potent than all-*trans* RA itself [15,18,19]. One explanation for the super potency of synthetic retinoids like Ro 13-7410 could be their slow metabolic clearance from sites of action. In fact, studies in the hamster indicate that Ro 13-7410 is cleared much more slowly than all-*trans* RA [20,21]. Taken together, these data suggest that binding affinities of retinoids for the receptors may not be completely predictive of the activities in vivo because of the complicating factors of metabolic activation and degradation. Alternatively, it is possible that synthetic retinoids might interact with retinoid receptors to produce a ligand receptor complex that is more active in inducing (or repressing) transcription than endogenous ligand receptor complexes, or the resistance of some retinoids to catabolism and differences in ligand receptor activity could explain some of the results of transcriptional activation studies in which some synthetic ligands induce receptor activity with greater potency (lower EC_{50}s) than all-*trans* RA [22–25].

Direct data to support the hypothesis that the activation of receptors is involved in the induction of toxicity comes from studies with a compound that selectively antagonizes RARα [26]. Experiments with this compound (Ro 41-5253) provide the most compelling evidence supporting the role of receptors in the toxic effects of retinoids. The myeloblastic leukemia cell line, HL-60 can be induced to differentiate to functional granulocytes by treating with various retinoids including the RAR-specific agonist, Ro 40-6055. When the HL-60 cells were treated with the combination of Ro 40-6055 and Ro 41-5253 there was an inhibition of differentiation [26]. The incidence of some of the teratogenic effects of the same RARα-selective ligand, was reduced

by cotreatment of mice with the antagonist Ro 41-5253 during days 8 and 9 of gestation (G. Schmidt unpublished results). Further direct evidence for the role of specific receptors in toxicologic phenomena will be forthcoming from studies with additional receptor-selective agonists and antagonists. From these studies it might be possible to associate specific biologic effects with the activity of specific receptor types. For example, activation of a specific receptor type in vivo would be expected to produce a response that is characteristic for that receptor. In this way, it will be possible to use pharmacologic intervention as a means of defining the role for each receptor in the various manifestations of retinoid toxicity.

The interactions of retinoid-ligands with their receptors results in receptor activation and ultimately in alterations in gene expression. It is now thought that these receptor-mediated changes in gene expression play a significant role in the toxicologic effects of retinoids.

How can a small molecule, like RA (or synthetic analogs), interact with a relatively few nuclear receptors to produce the marked physiologic and biochemical changes associated with toxicity or teratogenicity? Like the steroid hormones and their receptors, retinoids interact with receptors to initiate a cascade of changes in gene expression [3,4]. The first set of genes affected are those with expression directly modulated by retinoid receptors. The expression of these genes is controlled by the binding of the receptors to specific sequences of DNA (response elements) in the untranslated region of the gene. The interaction of a liganded receptor to its cognate response element results in activation (or repression) of the promoter regions of the gene. These early genes, referred to as primary genes, may themselves be transcription factors that control the expression of another set of genes which may in turn control the expression of yet another set of genes and so on. The cumulative result of this process is an amplification of the original retinoid signal through a cascade of gene expression changes. This type of gene expression cascade is analogous to the classic biochemical cascades associated with the binding of insulin or glucagon to their receptors, in which a biochemical signal at the receptor is amplified by subsequent biochemical processes of the cascade to produce a biologic response.

Data to support the existence of retinoid-induced cascades of gene expression come from studies on the differentiation of teratocarcinoma cell lines. These cell culture systems are models for differentiation and development. The effects of retinoids on these cells has been extensively studied and the changes in the patterns of gene expression between undifferentiated cells and differentiated cells has been well characterized [27]. Following RA treatment of F9 teratocarcinoma cells, one of the primary response genes is *Hoxa*-1 (formerly known as *Hox* 1.6), a member of the homeobox gene family. Expression of *Hoxa*-1 is transcriptionally regulated by RA treatment [28]. The homeobox genes are known to be important in development and differentiation of invertebrates and vertebrates. These genes contain a DNA-binding homeodomain and are thought to be transcription factors [29–31]. Thus the increased expression of *Hoxa*-1, in F9 cells is a potential initiating event in a gene cascade. The change in *Hoxa*-1 expression might be followed by changes in the expression of a number of genes that define the differentiated state of the F9 cells. Some of these genes encode for proteins that are structural like collagen and laminin while others encode for proteins that may control other transcriptional events [32,33]. In studies in human teratocarcinoma cell lines, the temporal induction of a homeobox gene cascade induced by RA treatment was investigated. Homeobox genes are arranged spatially on chromosomes in clusters. The most 3′ genes of the cluster are generally expressed earlier (and with boundaries of expression more anterior in embryos) than the genes at the 5′ end of the cluster [29]. These clusters of homeobox genes are turned on in a sequential manner by treatment with RA [34]. Since the homeobox genes are transcription factors, the end result of this cascade of transcription factors would be a secondary cascade of genes indirectly controlled by retinoid-receptor activation.

In whole animals and in particular in developing embryos, the induction of gene cascades can have profound effects. One of the most serious

toxicities produced by retinoid exposure is the malformation of developing embryos. Normal embryonic development is dependent on the presence of retinoids. Deficiency of vitamin A in pregnancy results in malformations and in extreme deficiency, preimplantation loss [35,36]. It is now thought that RA is an active factor in the control of development. Gradients of RA convey positional information for the normal development of the embryo [37]. RA signals are thought to convey the positional information by locally controlling gene expression [38–40]. Embryonic development can be thought of as a tightly controlled series of changes in gene expression in time and space. It has been well established that the exposure of vertebrate embryos to retinoids results in inappropriate gene expression patterns [41–44]. This would interfere with the normal patterns of gene expression in embryos thus explaining the marked morphologic changes associated with retinoid-induced teratogenesis. The relationships between the alterations in gene expression and the ultimate phenotype of the exposed embryo are being explored. Patterns of homeobox gene expression are now thought to encode information for the development of the vertebrae [45], the hind brain and the branchial arches [47,48]. One of these so called Hox codes contains the information for the specializations in the vertebrae [45]. Exposure of developing mice to RA is thought to change the Hox code and results in specific vertebral malformations [47,48]. If cascades of gene expression are important in retinoid teratogenesis, then direct manipulation of gene expression using transgenic animals should mimic some of the teratogenic phenotypes. In mice, overexpression of *Hoxa*-7 (formerly known as *Hox* 1.1) under the influence of the chicken β-actin promoter induces a phenotype with cranial and facial malformations that are reminiscent of some of the cranial and facial malformations associated with RA-induced teratogenesis [49]. Thus there is a growing body of literature supporting retinoid-induced changes in gene expression as a mechanism of developmental toxicity.

How, or if, receptor-mediated changes in gene expression relate to other toxic effects of retinoids needs to be explored further. Numerous studies at the biochemical and the molecular levels have demonstrated retinoid-induced changes in gene expression in epithelial cells in vitro and in vivo. In fact RA has been implicated as a necessary factor for epithelial differentiation. In retinoid-induced toxicity, the most commonly observed effects in both man and experimental animals are the mucocutaneous effects including erythema, epidermal thickening, and scaling [49–52]. The skin has a unique array of retinoid receptors, with high levels of expression of the RARγ [53] and the RXRα [6,53]. How the characteristic response of skin to retinoid exposure might be related to the presence of these receptor types is presently under investigation. These types of investigation will be aided by the development of receptor-specific agonists and antagonists.

There are a number of other adverse affects which are characteristic of hypervitaminosis A. The mechanism of these effects is under investigation at this time. There are now clear interactions of retinoid receptors with a number of different regulatory elements of a number of genes that can be related to some of the adverse effects of retinoids. For example, changes in serum lipids are a characteristic response to retinoid treatment. There is now evidence to suggest that RXR might have a role in regulating genes that control lipoprotein biosynthesis [9] and may control lipid metabolism through an RXR heterodimer [54] with the peroxisome proliferation activated receptor (PPAR) [55,56]. Thus, RXR-homodimers and RXR heterodimers might provide a mechanistic basis for retinoid-induced serum lipid changes. Similar heterodimeric interactions of RXR with the vitamin D and thyroid hormone receptors may also provide a mechanistic basis for understanding retinoid effects on bone and other tissues. The synthesis of receptor-selective ligands and the characterization of toxic effects of these selective ligands will elucidate the role of specific receptors in toxicity, while studies at the molecular level will identify gene pathways associated with toxicity.

In conclusion, it is now clear that the physiologic activity of retinoids is dependent on the

activation of specific receptors and receptor-mediated transcriptional regulation of various gene products. One of the challenges for retinoid toxicology is to obtain an understanding of the specific receptors and gene pathways that define toxicologic responses. The utilization of receptor-specific ligands to preferentially stimulate or block receptor types provides one approach for understanding retinoid activity at the level of the receptors. Transgenic animal experiments to knockout or to overexpress retinoid-associated gene products are being used to investigate gene pathways in retinoid activity. Utilization of these approaches will increase our understanding of retinoid toxicity. Understanding toxic mechanisms of action may lead to the development of less toxic and more potent therapeutic agents. However, there are additional benefits to be obtained from an increased understanding of retinoid toxicity. Retinoids are being used as tools by developmental biologists to perturb and probe developing organisms as a means to investigate the molecular processes of development and differentiation. In this context, it is possible that research on retinoid toxicity may have the most profound scientific influence.

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Pancreatic and nephrotoxicity of immunomodulator compounds

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Abstract

Increasing clinical experience of FK 506 in transplantation therapy has revealed a number of potentially restrictive adverse effects associated with its use. The mechanisms of action underlying 2 prominent toxic effects of FK 506, namely diabetogenesis and renal dysfunction, were investigated. A simple model system based on the effect of FK 506 on isolated rat pancreatic islets was utilised to study the relationship between inhibition of insulin biosynthesis, inhibition of interleukin 2 (IL-2) activation and FK binding protein (FKBP-12) binding of FK 506 and a number of FK 506 analogues. Results indicate that the action of these compounds on inhibition of insulin biosynthesis (and by implication, diabetogenesis) may be related to their immunosuppressive potential. Observations on the FK 506-induced release of endothelin-1 from isolated rat kidney mesangial cells suggest that this cell may be an important target associated with the nephrotoxic potential of the drug, and that this action may be mediated via the FKBP.

Keywords: FK 506; Immunosuppressants; Nephrotoxicity; Diabetogenesis; Insulin biosynthesis; Structure-activity relationships

1. Introduction

The fungal macrolide antibiotic, FK 506, possesses potent immunosuppressive activity and has been successfully used both experimentally and clinically to prevent organ graft rejection. However, although initial experience with FK 506 suggested that it may have a reduced spectrum of toxicity compared to cyclosporin A (CsA), an established therapeutic agent with related immunosuppressive action, increasing clinical use has revealed a number of similar side-effects, prominent amongst which are diabetogenesis and renal dysfunction [1]. The incidence of diabetogenesis encountered in recent clinical trials of FK 506 carried out in kidney transplant patients was 16–47% [1–3]. An undesirable consequence is that insulin therapy may be required to treat the FK 506-induced diabetogenesis [4]. Renal dysfunction appears to be the primary dose-limiting toxic effect of FK 506, with an incidence of 27.1–44.4% in renal transplant patients [2,3]. Principal functional changes include a reduction in glomerular filtration rate (GFR) and renal blood flow (RBF) [5].

The mechanisms underlying FK 506-induced diabetogenesis and renal dysfunction are not fully elucidated, although clearly such a toxicity profile is undesirable and may limit its use in the clinic. The development of alternative less toxic

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structures based upon the parent FK 506 molecule would undoubtedly be facilitated by a greater understanding of the mechanism of FK 506-induced toxicity and the development of toxicity screens that would enable FK 506 analogues to be assessed and compared alongside data from established efficacy screens. Practical criteria for such toxicity screens would ideally incorporate quantitative and rapid end-points together with low drug usage and would probably be best served by in-vitro test systems. An essential prerequisite for such a strategy would be to demonstrate relevance of the screens to the clinical profile of FK 506 toxicity. Consequently, we have undertaken a series of studies to investigate the diabetogenic and nephrotoxic properties of FK 506, with the objective of providing additional evidence on its mechanism of toxicity and subsequently developing potential toxicity screening tests.

2. FK 506-induced diabetogenesis

The diabetogenic effect of FK 506 generally manifests as hyperglycaemia after repeated dosing in experimental animals. Rats, dogs and primates have been shown to be susceptible to this effect (in-house data). In a study carried out in the Sprague–Dawley rat, FK 506 administered orally for 5–10 days at 15 $mg \cdot kg^{-1} \cdot day^{-1}$ significantly reduced tolerance to an oral glucose load (see Fig. 1). This was accompanied by a depressed post-load plasma insulin response, suggesting that the glucose intolerance may have been associated with an inappropriate insulin secretion. Follow-up investigations, using immunocytochemistry for localisation of insulin immunoreactivity and in-situ hybridisation histochemistry to localise insulin mRNA in the pancreas, showed that FK 506 dosing resulted in a marked reduction of both insulin parameters. Dot-blot analysis using ^{32}P-labelled cDNA probes confirmed a selective reduction in pancreatic insulin mRNA levels (relative to β-actin) after 5 or 12 days dosing at 10 and 15 $mg \cdot kg^{-1}$ FK 506 (see Fig. 2). FK 506-induced glucose intolerance therefore appears to be associated

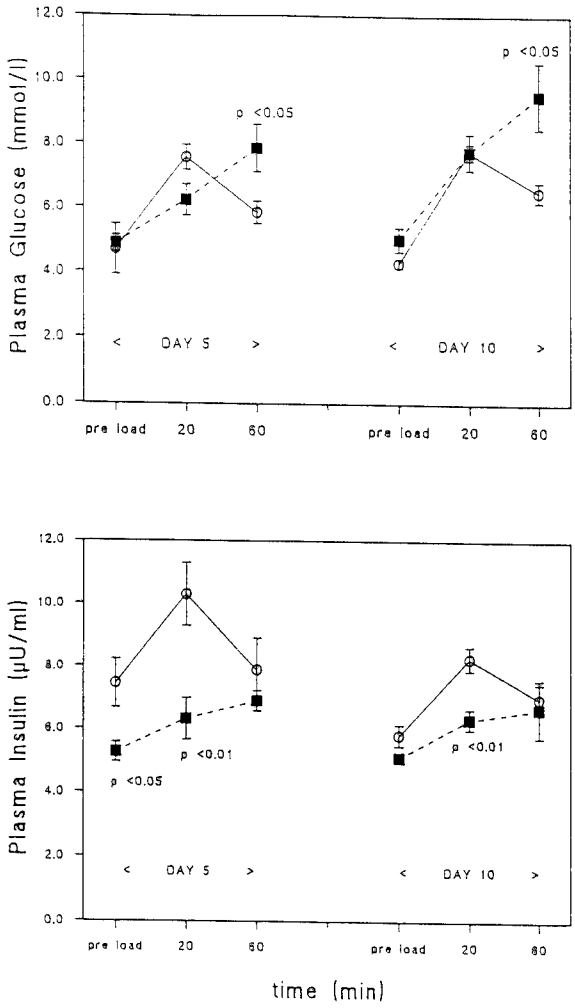


Fig. 1. Effect of 15 $mg \cdot kg^{-1} \cdot day^{-1}$ FK 506 on oral glucose tolerance. Glucose tolerance was measured in male Sprague–Dawley rats (7 animals per group) after 5 and 10 days dosing (○, control group; ■, FK 506 dosed group. Bars = S.E.M.).

with decrease in pancreatic insulin gene expression, resulting in depletion of pancreatic insulin content. These data confirm the hypothesis that the hyperglycaemic action of FK 506 occurs via inhibition of pancreatic islet function [6,7].

2.1. Effect of FK 506 on isolated rat islets

In order to utilise the potential for FK 506 to directly affect insulin biosynthesis by pancreatic islet cells, in vitro studies were performed on rat islets isolated and maintained in culture for up to

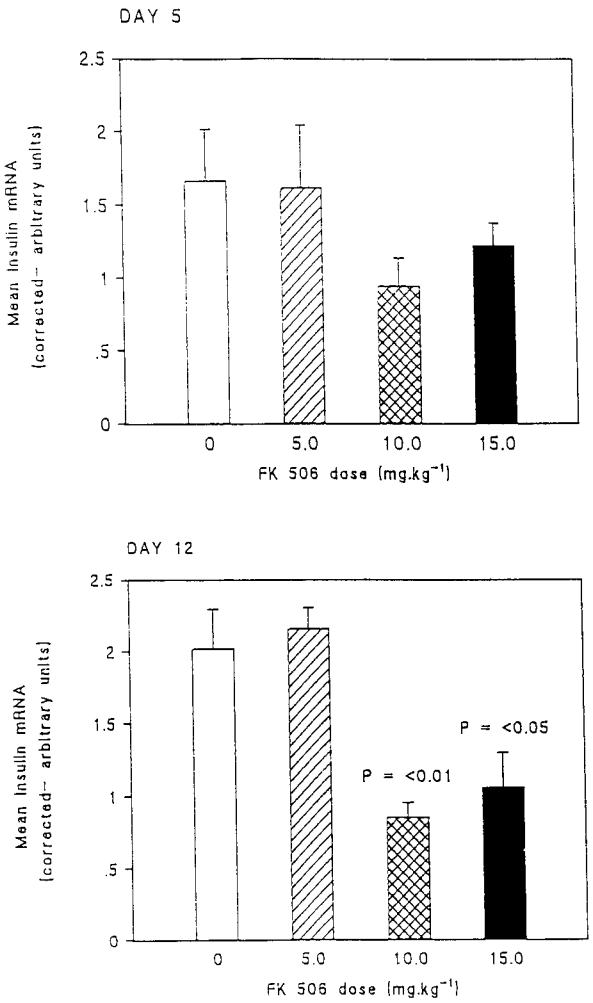


Fig. 2. Effect of FK 506 on relative pancreatic insulin mRNA levels in the rat (6 animals per group). Bars = S.E.M.

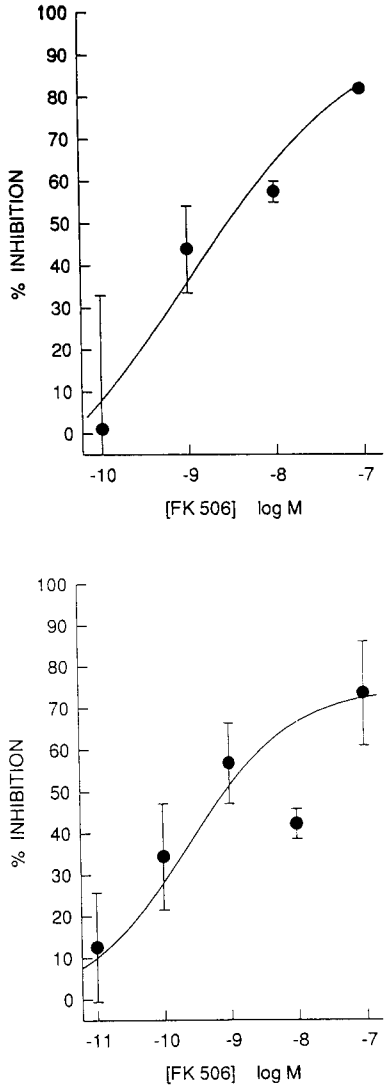


Fig. 3. (a) Graph showing the effect of increasing FK 506 concentration on inhibition of insulin peptide biosynthesis in vitro. Mean percentage inhibitions were calculated from 5 separate experiments and the line of best fit estimated using 4 parameter logistic fit to describe the drug dose-response curve. Bars = S.E.M. (b) Graph showing the effect of increasing FK 506 concentration on inhibition of insulin mRNA in vitro. Mean percentage inhibitions were calculated from 3 separate experiments and the line of best fit estimated using 4 parameter logistic fit to describe the drug dose-response curve. Bars = S.E.M.

6 days in the presence or absence of the drug over the range 10^{-6}–10^{-11} M. Insulin mRNA content of cultured islet preparations was measured by dot-blot analysis as above. Insulin peptide synthesis was estimated by pulse labelling with [^{35}S]cysteine followed by immunoprecipitation and SDS/PAG electrophoresis.

FK 506 treatment resulted in a pronounced dose-related reduction of insulin mRNA levels in cultured islets, with maximal inhibition of 90% and an estimated IC_{50} of 1.5×10^{-9} M (see Fig. 3a). Evidence of reduced mRNA levels was apparent from 1 day's exposure onwards. Insulin biosynthesis was also severely decreased following exposure of islets to FK 506, although maximal inhibition was somewhat less (approximately

75%), with an IC_{50} of 3×10^{-10} M FK 506 (see Fig. 3b). In contrast to mRNA levels, insulin biosynthesis was not affected after 1 day's exposure but was clearly impaired from 3 days' exposure onwards, suggesting that the initial effect of FK 506 may be upon insulin mRNA. These in vitro data show that FK 506 is a potent and direct inhibitor of insulin production within the pancreatic islet and provide strong support for in vivo findings, indicating that insulin inhibition is most likely to be the primary cause of the diabetogenic action of FK 506. Further work would be required in order to determine the point of action of FK 506 on insulin gene expression (e.g. transcriptional block).

2.2. Effect of FK 506 analogues on insulin inhibition: correlation with FK binding and immunosuppressant activity

The relationship between insulin inhibition (and by implication, diabetogenic potential) and immunosuppressant activity of FK 506 was explored using a series of structural FK 506 analogues. These, together with the immunosuppressants CsA and Rapamycin (Rap) were tested for inhibition of insulin biosynthesis using the isolated rat islet culture system as described above. The immunosuppressive action of FK 506 is thought to occur via binding to a specific immunophilin, the FK binding protein (FKBP-12), and subsequent inhibition of calcineurin-dependent T cell activation pathways by the FK 506-FKBP complex [8]. FKBP binding and calcineurin inhibition appear to be determined by separate domains of the FK 506 molecule, but both are required for immunosuppressive activity. Data obtained previously for inhibition of interleukin 2 (IL-2) activation in Jurkat cells (a measure of immunosuppression potency) and FKBP-12 binding [9] was plotted against insulin inhibition data (see Fig. 4). These show that analogues able to bind to FKBP-12 and inhibit IL-2 production are in general also inhibitors of insulin biosynthesis. Analogues of FK 506 (including Rap and FPL 65620), that bind to FKBP-12 but are poor inhibitors of IL-2 production, were found also to be poor inhibitors of insulin biosynthesis. Thus, it seems likely that the efficacy (IL-2 inhibition) and toxicity (insulin inhibition) of FK 506 and related compounds are in some way associated, presumably via a related pathway involving FKBP binding. However, one FK 506 analogue (labelled 'X'), was found to have moderate FKBP-12 binding and IL-2 inhibitory activity, but poor insulin inhibition, and it is therefore possible that additional and as yet unidentified factors may be important determinants in these complex pathways, maintaining the possibility that the immunosuppressant and pancreatic toxicity of FK 506 structures may be separated. Areas for further investigation include differential distribution and involvement of

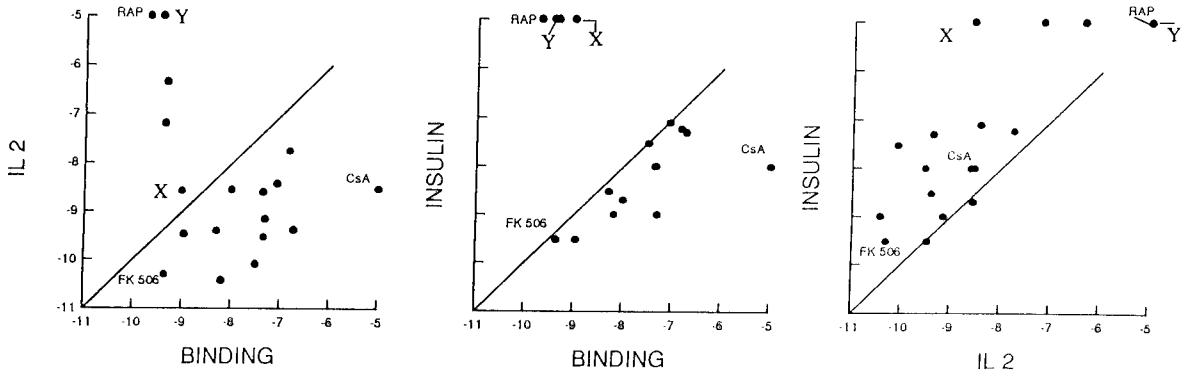


Fig. 4. Correlation curves for IL-2 inhibition, FKBP-12 binding and in vitro inhibition of insulin biosynthesis by FK 506 analogues (all points shown are $\log IC_{50}$). X denotes an analogue with moderate IL-2 inhibition and FKBP binding affinity, Y denotes FPL 65620.

FKBP and calcineurin subtypes in target tissue signalling pathways, together with receptor binding selectivity and potency of FK 506 analogues.

3. FK 506-induced nephrotoxicity

Cumulative clinical and experimental data indicate that the nephrotoxic action of FK 506 is mediated via perturbation of renal haemodynamics, resulting in a reduced GFR, decreased RBF, and ultimately, functional deficit. The mechanism underlying this toxicity, however, remains unresolved, although many of its features appear similar to that seen with CsA [5]. In fact FK 506-induced renal dysfunction, as measured by standard parameters (e.g. plasma creatinine increase), is not readily attained in most strains of rat tested, presumably due to their large renal functional reserve. This has led to difficulty in the assessment of FK 506 toxicity. Nevertheless, various renal changes are seen in the FK 506 dosed rat that seem to reflect its mode of action. Studies carried out in Sprague–Dawley rats dosed orally with FK 506 at 1–9 $mg \cdot kg^{-1}$ for up to 14 days revealed a profound dose-related increase in juxtaglomerular (JG) renin mRNA content (up to 350% renin mRNA positive JG compared to controls) and a corresponding elevation of plasma renin activity (2–3-fold increase over controls). Afferent arteriolar smooth muscle cells were also found to be recruited into renin expression in a pattern similar to that observed with angiotensin-converting enzyme (ACE) inhibitors. When tested, CsA produced an identical pattern of renin expression to FK 506 in the rat. The observed upregulation of renin expression due to FK 506 would, unlike ACE inhibitors, result in an increase in angiotensin II-mediated renal vasoconstriction. However, this is likely to reflect a secondary compensatory response to a pre-existing effect rather than the primary mechanism of FK 506-induced changes in GFR, as the principle effect of angiotensin II would be to increase efferent arteriolar resistance, and maintain filtration pressure. The similarity between FK 506 and CsA effects on renal renin expression are consistent with a common mechanism of action. When tested, the FK 506 analogue FPL 65620, which has poor immunosuppressive activity, showed no effect on renal renin expression in the rat, suggesting a possible link between efficacy and nephrotoxicity potential.

Further evidence of the potential renal vascular effect of FK 506 was obtained from morphometric analysis of rat kidney sections following oral dosing with 3–15 $mg \cdot kg^{-1}$ FK 506 for 7 days. This produced a dose-related reduction in glomerular mean cross-sectional area (maximum 20% decrease at 9 $mg \cdot kg^{-1}$ FK 506 compared to controls). Dosing with CsA at 27 and 81 $mg \cdot kg^{-1}$ CsA for 7 days produced similar changes. Work carried out by ourselves (data not shown) and others [10] on isolated rat glomerulus preparations in vitro suggests that FK 506 and CsA may be capable of causing a direct glomerular contraction. Further in vitro experiments were consequently carried out to identify possible effects of FK 506 on the principle contractile element of the glomerulus, the mesangial cell.

3.1. In vitro effect of FK 506 on release of endothelin-1 from rat mesangial cells

The potent vasoconstrictive peptide, endothelin-1 (ET-1) plays an important role in the regulation of renal haemodynamics [11] and a number of cell types within the kidney, including the mesangial cell, are known to be capable of its synthesis and release. Recent work on CsA and FK 506 suggests that these drugs may cause an alteration of ET-1 activity in vivo [12,13]. Blockade of ET-1 receptors using specific antibodies or selective antagonists has been shown to attenuate FK 506 and CsA-induced renal dysfunction [14,15]. ET-1 may therefore be a key component in the mechanism of nephrotoxic action of the immunosuppressants.

Studies carried out on isolated rat mesangial cells in culture showed a clear potential for FK 506 and CsA to cause a direct increase in the release of ET-1 from rat mesangial cells when added to cultures for 6–24 h. Maximum ET-1 release (approximately 2.75 times basal level) was produced at an FK 506 concentration of 10^{-8} M (see Fig. 5), i.e. generally consistent with

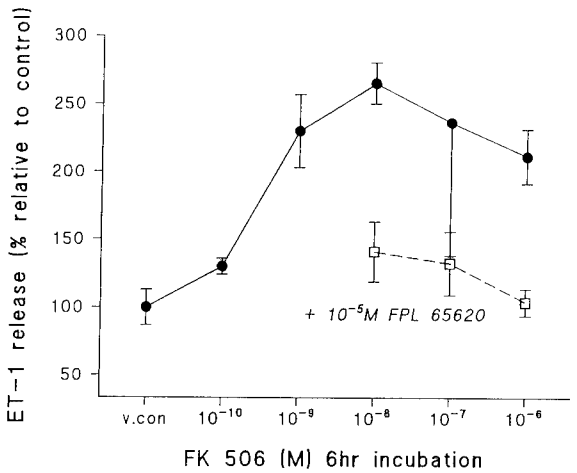


Fig. 5. Graph showing the effects of increasing FK 506 concentration on release of ET-1 from sub-confluent mesangial cells in vitro (●). Results are expressed as mean percentage ET-1 release relative to control. Bars = S.E.M. ET-1 release was blocked by co-incubation with FPL 65620 (□), an FK 506 analogue.

trough FK 506 blood levels associated with renal dysfunction [16]. ET-1 released from mesangial cells (and possibly other cell types, including endothelial cells [17]) in response to FK 506 exposure may act locally via ET-1 receptors within the kidney to influence renal haemodynamics. The fact that mesangial cells themselves possess ET-1 receptors gives rise to the possibility of an autocrine pathway for FK 506 action. The effect of FK 506 on mesangial ET-1 release was shown to be blocked by addition of FPL 65620, suggesting that this FK 506 action (as with its diabetogenic effect) occurs via binding to the FKBP. Although links between FK 506, mesangial ET-1 and in vivo renal dysfunction remain to be proven and relevance to the clinical situation established, these data indicate that further work on this model would be justified in order to fully assess its merit as a screen for the nephrotoxic potential of FK 506 analogues.

4. Discussion

The potent inhibitory action of FK 506 on pancreatic insulin synthesis and the insulin-associated glucose intolerance demonstrated by FK 506 in experimental models is consistent with its diabetogenic effect observed in the clinic. Although unequivocal evidence of insulin inhibition as the functional target for FK 506-induced diabetogenesis is presently lacking from the clinic, experimental findings indicate that this is highly probable. Data from the in vitro islet studies carried out on FK 506 analogues indicate that the action of FK 506 on insulin may have mechanistic parallels with its immunosuppressive effect. Scope for separation of efficacy and toxicity (with respect to diabetogenic potential) would therefore appear to be limited, although deviations observed for some FK 506 analogues maintain the possibility for selectivity. Selectivity would undoubtedly be facilitated by a greater understanding of the role of FKBP/calcineurin involvement in the control of insulin biosynthesis.

The detailed mechanism responsible for the nephrotoxic action of FK 506 is not yet established. Observations on the effect of FK 506 on ET-1 release from mesangial cells in vitro are consistent with a vascular/haemodynamic mode of action, although further work is required in order to establish the relevance and significance of this finding. The effect of FPL 65620 in blocking ET-1 release by FK 506 does however indicate that the mesangial cell action of FK 506 is FKBP-dependent and therefore probably associated with its immunosuppressive mode of action. A link between nephrotoxicity and immunosuppression is further indicated by the increasingly apparent similarities between FK 506 and CsA effects in the clinic. A number of different mechanisms and targets have been suggested to explain FK 506-induced nephrotoxicity reflecting both the complexity and multiplicity of systems controlling renal homeostasis. This is further complicated by the wide tissue distribution of the FKBP, effectively increasing the number of potential targets for FK 506 action. Indeed, it seems probable that the renal dysfunction caused by the drug may be the combined result of a number of individual effects.

Although the results of these studies tend to

diminish the prospect of improving the therapeutic ratio of FK 506-based compounds through differential reduction of its toxicity profile, mechanistic strategies based upon identification and utilization of the target toxicity for correlation with efficacy parameters have clearly been shown to have value in structure-activity based drug discovey.

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Cultured hepatocytes as investigational models for hepatic toxicity: practical applications in drug discovery and development

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Abstract

Drugs can fail at any phase during discovery, preclinical or clinical development due to unacceptable levels of toxicity, and liver is commonly the principle target organ. Investigational toxicology methods, using appropriate models and hypotheses, can often resolve problems, identify toxic chemical substituents and salvage therapeutic discovery programs. While in vivo models are used to investigate hepatic drug effects in the context of toxicokinetics and systemic influences, cell culture models provide in vitro systems for investigating specific mechanisms in a precisely controlled environment. Using primary hepatocytes isolated from laboratory animals, we have explored several drug-induced hepatic disorders that surfaced during different phases of drug discovery and development. Additionally, the use of human hepatocytes has allowed us to address concerns for human exposure, examine human relevance of animal data, and provide perspective on problems encountered in clinical trials.

Keywords: Liver; Hepatotoxicity; Hepatocyte; Drug; In vitro

1. Introduction

The drug discovery and development process consists of a series of investigational phases, beginning with the demonstration of efficacy in experimental cell and animal models and concluding with the demonstration of safety and efficacy in humans. This process is long and often protracted by unanticipated problems. Drugs can fail at any point, and failure at later times is inevitably more costly. While failure can be due to a variety of factors (including, for example, business and manufacturing considerations), it is often due to unacceptable toxicity in one or more animal species or in clinical trials. By contributing to the understanding of a toxicity and providing perspective, investigational toxicology programs can enhance the discovery and development process. Investigational toxicology efforts can provide information regarding toxic mechanisms of a drug, and models for avoiding repeated toxicity problems. For toxicity revealed during preclinical development, determining the mechanism and providing an investigational

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model can contribute to the discovery or design of a less toxic analog. Alternatively, mechanistic studies can place the observed toxicity in perspective in regards to human risk. Unfortunately, not all problems can be anticipated during preclinical development, and toxicity can be encountered during the clinical trial phase. In this situation, investigational techniques can help identify the biological mechanism of toxicity in regard to species differences (and lack of predictability), and perhaps salvage a chemical series with promising pharmacology and its corresponding discovery program by identifying the responsible chemical moiety.

In mechanistic problem solving, it is important to select appropriate investigational models. While in vivo models are needed to investigate drug effects in the context of toxicokinetics and systemic influences, in vitro models can be efficient and cost-effective tools for investigating specific mechanisms in a precisely controlled environment. In vitro models are generally target-organ based, and the most frequently encountered target-organ toxicity is liver. This is due to a variety of reasons but is generally related to drug clearance and metabolism. Three experimental models are in common use for studying hepatic toxicity: perfused liver, precision-cut slices and isolated cells (usually hepatocytes). Each model has its advantages and disadvantages, and model selection should be based on the specific problem to be resolved. In this report we discuss the utility of primary cultured hepatocytes isolated from a variety of species as models for investigating drug-induced hepatic toxicity, and the incorporation of these models into various phases of the drug discovery and development process. Cultured rat hepatocytes and other cell models have been used for many years to study toxicology in vitro (reviewed in [1]), and the application of cultured hepatocytes, particularly rodent, for examining hepatic xenobiotic toxicity and metabolism has been the subject of books (i.e., [2]). Cultured human hepatocytes have facilitated the evaluation of human responses relative to other animal species. For example, cultured human hepatocytes have been used to examine human drug

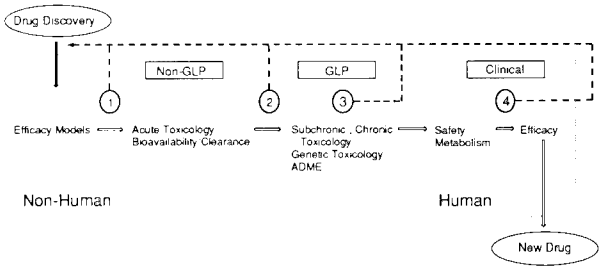


Fig. 1. Applications of investigative toxicology studies using cultured hepatocytes in the drug discovery and development process. Cultured hepatocyte models have been used at several points in preclinical (non-human) and clinical (human) drug development. (1) Screening drugs that exhibit hepatic toxicity or other hepatic effects for lead selection. (2) Mechanistic studies to address specific concerns (i.e., peroxisome proliferation or hepatic steatosis) and metabolism studies for animal toxicology species selection. (3). Mechanistic studies to resolve hepatic toxicity problems, provide potential human relevance and clinical markers. (4) Investigational studies to determine mechanism for hepatic toxicity observed in human clinical trials. At all phases, feedback is provided to the drug discovery effort.

metabolism [3], genotoxic potential of chemicals towards human liver [4], and relevance of animal toxicology findings to humans [5]. Cultured hepatocytes, as models for investigative toxicology, have moved well beyond the experimental stage and have become incorporated as key components in the drug discovery and development process (Fig. 1).

2. Hepatocyte isolation, culture and cryopreservation

Rat hepatocyte isolation protocols have in general been based on the 2-step collagenase perfusion devised by Seglen [6]. This perfusion technique was modified and adapted for isolating monkey hepatocytes [7], a procedure that has also worked well for isolating hepatocytes from other species (rabbit, dog, pig and human). For short-term culture (up to 4 days), isolated hepatocytes are plated on collagen substrates, although rat hepatocytes will attach to uncoated tissue culture plastic. For long-term culture it is necessary to provide hepatocytes with the signals needed to maintain differentiated functions. This

is accomplished by a variety of methods including (but not limited to) co-culture with epithelial cells [8], addition of basement membrane (matrigel; [9]), or culture in a collagen sandwich [10].

The utility of hepatocytes isolated from an individual donor can be extended by cold storage and cryopreservation. Cold storage techniques extend the useful window to about 48 h post-isolation with little loss in viability [11], while cryopreservation can extend this period indefinitely [12]. The development of successful methods for cryopreservation has lagged behind progress in isolation and culture techniques, however, and viability of stored cells is inevitably much lower than freshly isolated or cold-stored cells. Loss of viability is due to several factors including initial cell integrity, hypoxia during freezing, ice crystal formation during or after freezing, and toxicity of cryopreservation medium components. For human, the most difficult factor to control is the initial cell integrity. While viability following isolation may be high (90–95%), cells may be compromised by an extended time period between organ cross-clamp and cell isolation procedures. In our experience, the best human hepatocytes for cryopreservation are obtained from donor tissue with minimal transport time (less than 24 h) and minimal hepatocellular fat content. Hypoxia during freezing can be reduced by periodic inversion of the storage vials (C. Chesne and A. Guillouzo, pers. commun.), though this is difficult if an automated cell freezer is used. Ice crystal damage can be minimized by controlled freezing at a rate of -1°C/min with special care at the freezing point, since this is where damage is most likely to occur. Temperature records from automated freezers are quite useful for monitoring specimen warming, and programs can be adjusted accordingly. Frozen cells should be stored in liquid nitrogen to prevent ice recrystalization. Most freezing media contain serum (10–90%) and a cryoprotectant such as DMSO (10–15%). At these concentrations DMSO can be toxic, thus cells should be rinsed as soon as possible after rapid thawing. Even under the best of conditions, plating efficiency is reduced by about 50% for most species [12]. We use a protocol developed for monkey hepatocyte cryopreservation [13] to freeze human hepatocytes. A summary of recovery viability and plating efficiencies is presented in Table 1.

3. Drug metabolism by cultured hepatocytes

One principle reason for using hepatocytes for in vitro toxicology studies as opposed to other cultured cell types (i.e., hepatoma cell lines) is their capacity for drug biotransformation. Cultured hepatocytes are known to lose cytochrome P450 activities with time compared to in vivo levels. Metabolizing capacity can be maintained or induced by addition of other matrix materials such as matrigel [9,14] or a specific cytochrome activity can be induced with the appropriate inducing agent, such as 4A1 and 2B1 induction by clofibrate [15]. However, sufficient metabolizing capacity does remain over the first few days in culture on collagen for many applications. This is demonstrated by the experiments summarized in Fig. 2 and Table 2. The triazoloben-

Table 1
Hepatocyte viability and plating efficiency following cryopreservation

	Viability before freezing	Viability after freezing	Viable cells plated	Cells attached	Plating efficiency[a]
Example (monkey)	93%	85%	$1.1 \times 10^5/cm^2$	$5 \times 10^4/cm^2$	45%
Range (monkey)	90–98%	80–90%			40–65%
Range (human)	85–95%	75–85%			40–65%

The example is from a single monkey hepatocyte isolation and cryopreservation study; the ranges for monkey and human are summarized from several (>15) isolations. Viability was determined by trypan blue dye exclusion, and plating efficiency was determined by cell counting.

[a] Percent of viable cells that attached to plates.

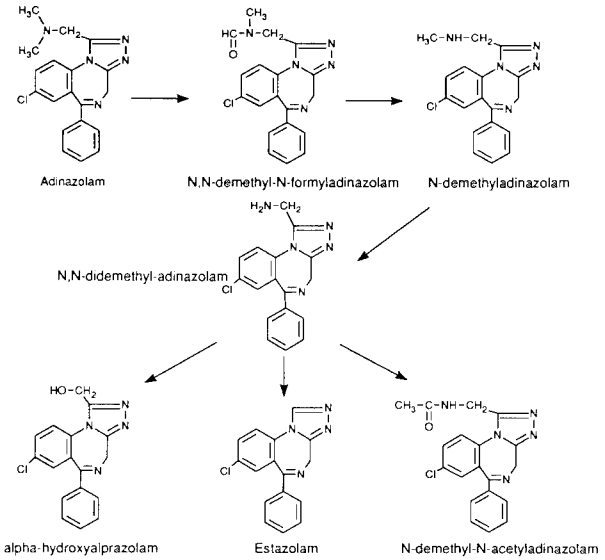


Fig. 2. Proposed metabolic path of adinazolam in humans.

zodiazepine, adinazolam, is extensively metabolized by many species including humans [16]. The proposed metabolic route and resultant human metabolite structures are presented in Fig. 2. One day (24 h) after isolation, cultured hepatocytes from human, monkey, dog and rat on collagen-coated dishes were exposed to adinazolam at a concentration of 15 μg/ml for a 24-h period. Culture media were then analyzed by HPLC for concentrations of parent drug and metabolites. In general, the metabolites formed in vitro correlated well with those formed in vivo (Table 2), with clear demonstration of species-specific metabolites. Also included for comparison is the metabolite profile obtained with rat S9, which produced a somewhat different pattern of metabolism from rats or rat hepatocytes. The data presented indicate that cultured hepatocytes maintain the capacity to metabolize this drug at

Table 2
A comparison of adinazolam and metabolites detected in plasma and urine from animals in vivo and the corresponding hepatocyte cultures in vitro

Species	Source	Parent drug and metabolites								
		ADM	DMFA	DMA	DDMA	DDAA	αHA	EST	αDHA	DHMA
Human	Plasma	*Mj	*Tr	*Mj	*Mj	*	*	*		
	Urine	*	*	*Mj	*Tr	*Tr	*	*		
	Hepatocytes	*Mj		*Mj	*		*Mj	*Tr		
Monkey	Plasma	*Mj	*	*Mj	*Mj	*	*	*		
	Urine	*	*Tr	*Mj	*Tr	*Tr	*Tr[a]	*Tr	*Tr	*Tr
	Hepatocytes	*Mj	*Tr	*Mj	*Mj	*	*	*Tr		
Dog	Plasma	*Mj		*Mj	*Mj		*Mj	*Mj	*	
	Urine	*	*	*	*		*Mj	*Mj	*	
	Hepatocytes	*Mj	*Tr	*Mj	*		*Mj	*Tr		
Rat	Plasma	*Mj		*	*					
	Urine	*		*	*		*	*Mj	*Mj	
	Hepatocytes	*Mj	*Tr	*Mj	*Mj		*Tr	*Tr		
	Rat S9	*Mj		*			*Tr[a,b]	*Tr	*Tr	

[a] Identified from urine after hydrolysis of glucuronide or sulfate.
[b] Questionable identity of metabolite identified by TLC.
* Asterisk indicates detection of a drug-related metabolite.
ADM, adinazolam; DMFA, *N*-demethyl-*N*-formyladinazolam; DMA, *N*-demethyladinazolam; DDMA, *N*,*N*-didemethyladinazolam; DDAA, *N*,*N*-didemethyl-*N*-acetyladinazolam; αHA, α-hydroxyalprazolam; EST, estazolam; αDHA, α,4-dihydroxyalprazolam; DHMA, α,4′-dihydroxymethylalprazolam. Mj, major identifiable drug-related compound at concentration >10% of metabolites formed; Tr indicates trace identifiable drug-related compound at concentration <1.0% of metabolites formed.

least for the first few days in culture. In practical use, such drug metabolism profiles can be used prospectively to help select the laboratory species that produce a metabolic profile most similar to humans to use in preclinical toxicology studies. Alternatively, if toxicity is observed in one animal species, metabolic profiles generated by cultured hepatocytes can be used to identify potential toxic metabolites and determine potential human exposure.

4. Applications in investigative toxicology

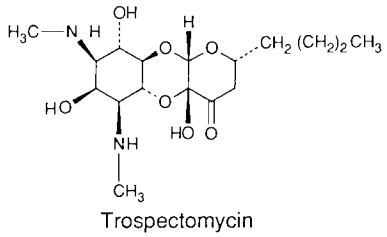
In practice, we can rarely determine the precise mechanism of toxicity for any drug but can establish sufficient surrogate models and assays to aid discovery and development efforts. To investigate mechanism and provide in vitro models to support discovery efforts, a combination of cytotoxicity and cell functional assays are developed. When evidence of hepatic toxicity is first observed, the immediate task is to determine the sensitive species; this may require limited additional in vivo studies. For example, to investigate hepatic toxicity observed in the dog, but not the rat, dog hepatocytes would be the initial species to examine as a potential in vitro model. It is then important to establish assay endpoints to be used in vitro that mimic aspects of the toxicity observed in vivo. Cytotoxicity (cell lysis) assays such as lactate dehydrogenase (LDH) release or propidium iodide uptake are useful for determining relative sensitivity between species (including human), and whether hepatic toxicity can be attributed to direct drug exposure. These assays are employed early in the investigational process, and may provide clues as to whether or not drug metabolism is prerequisite to cell death, or if cell death may be due to intrinsic levels of parent drug. Generally, the utility of cytotoxicity assays is limited to establishing drug exposure times and concentrations that are not cytolytic and may be used to further investigate cell physiology.

Detecting cell physiological changes in vitro that are reflective of cell physiological changes in vivo is an important step in determining biological mechanism. Most drugs do not produce hepatic toxicity directly but rather work by perturbing essential physiological processes, such as inhibiting ATP synthesis or ion transport. While this may eventually lead to cell death (likely through apoptosis as opposed to oncosis), cells in culture may not die in the presence of physiologically relevant concentrations of drug. It is therefore necessary to establish cellular markers (assay endpoints) that are sensitive to physiological or structural changes that precede cell death. Clues as to the appropriate cell functional markers to investigate are obtained from careful examination of existing animal data, which may include hepatic structural alterations (fat deposition, mitochondrial swelling, endoplasmic reticulum or peroxisomal proliferation), functional alterations (changes in blood glucose or albumin) and enzymatic alterations (changes in cytochrome P450, lysosomal or peroxisomal enzyme activities). It is apparent that the particular assays employed in vitro are thus as varied as the biological mechanisms of toxicity produced by drugs in whole animals and are best explored by example.

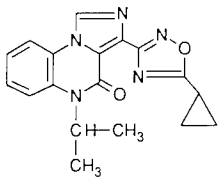
The following discussions summarize studies concerning 2 different drugs that produced investigative toxicology challenges at different phases of drug development. The first, trospectomycin, provides an example of a toxicology problem that surfaced during preclinical development studies. The second, panadiplon, is an example of a problem that occurred during clinical development. In both cases, investigational models were provided to discovery program chemistry efforts.

5. Trospectomycin: hepatic toxicity observed during preclinical development

Trospectomycin (Fig. 3) is a highly water-soluble, broad-spectrum antibiotic with improved antimicrobial activity over spectinomycin [17]. During preclinical development it was found to produce a dose- and time-dependent increase in serum ALT and AST values in rats and dogs, along with a coincident and reversible appear-



Trospectomycin



Panadiplon



Cyclopropane carboxylic acid

Fig. 3. Chemical structures for the antibiotic trospectomycin (U-63366F; 6′-*n*-propylspectinomycin hydrogen sulfate, pentahydrate), the quinoxalinone anxiolytic panadiplon (U-78875; 3-[5-cyclopropyl-1,2,4-oxadiazol-3yl]-5-[1-methylethyl]-imidazo{1,5-a}-quinoxalin-4[5H]-one), and a metabolite of panadiplon, CPCA.

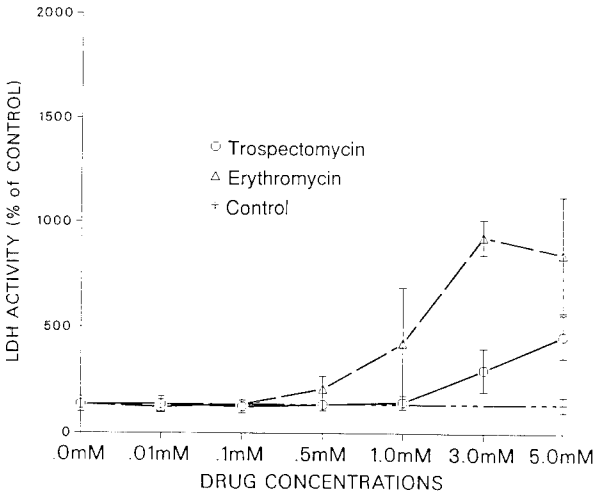


Fig. 4. Cytotoxicity of the antibiotic, trospectomycin, compared to erythromycin in cultured rat hepatocytes. Hepatocytes were incubated for 24 h in the presence of drugs, and cytotoxicity was determined by LDH present in medium. Results are expressed as percent control values for paired control cultures.

ance of hepatocellular lamellar inclusion bodies and phospholipidosis [18,19]. To investigate the potential mechanistic role that phospholipid accumulation may play in producing cell death, we conducted experiments with cultured rat hepatocytes and the perfused rat liver model. Endpoints selected for these studies included cell lytic assays (enzyme release) and lamellar body formation (as a marker for phospholipidosis). The results of these experiments clearly differentiated lamellar body induction from cytotoxic activity [20,21], indicating that phospholipidosis does not directly or necessarily result in toxicity. Further, examination of structural analogues indicated that increased alkyl side chain length was responsible for increased phospholipid storage and toxicity [22], providing a mechanistic model to synthetic chemistry efforts. Phospholipidosis potential determination has been further enhanced by the development of a fluorescence technique [23].

Cultured hepatocytes from rat, monkey and human were also used to estimate relative or potential human risk. In these experiments, hepatocytes were plated on collagen and, following overnight incubation, exposed to several concentrations of trospectomycin for 24–48 h. Cells were examined for cytotoxicity by LDH release [24] and lamellar body formation by electron microscopy. Trospectomycin produced cytotoxicity at concentrations >1.0 mM in Sprague–Dawley rat hepatocytes (Fig. 4); significant lamellar body induction was observed at 1 mM [22]. Monkey and human hepatocytes, however, showed no evidence of cytotoxicity or lamellar body induction at concentrations up to and including 5.0 mM (Figs. 5 and 6), even after 48 h continuous exposure. By comparison, erythromycin (base) produced significant cytotoxicity at concentrations of 0.5 mM and above in rat and monkey hepatocytes. These experiments indicate that humans and monkeys are more resistant to trospectomycin-induced toxicity and phospholipidosis than rat. The relevance of direct drug exposure in this case is supported by the minimal metabolism of trospectomycin [25].

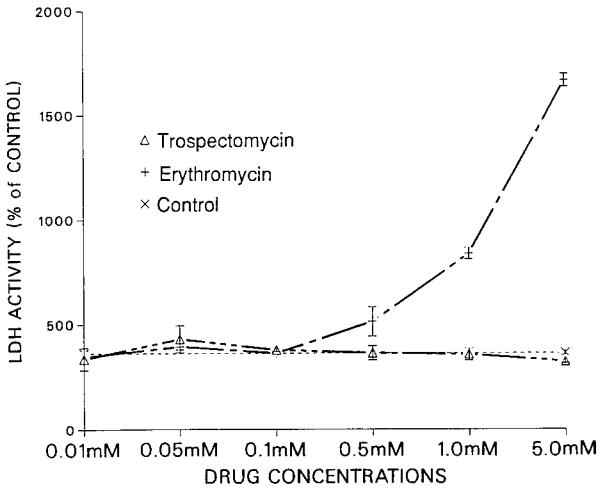


Fig. 5. Cytotoxicity of the antibiotic, trospectomycin, compared to erythromycin in cultured monkey (*Macaca fascicularis*) hepatocytes. Hepatocytes were incubated for 24 h in the presence of drugs, and cytotoxicity was determined by LDH present in medium. Results are expressed as percent control values for paired control cultures.

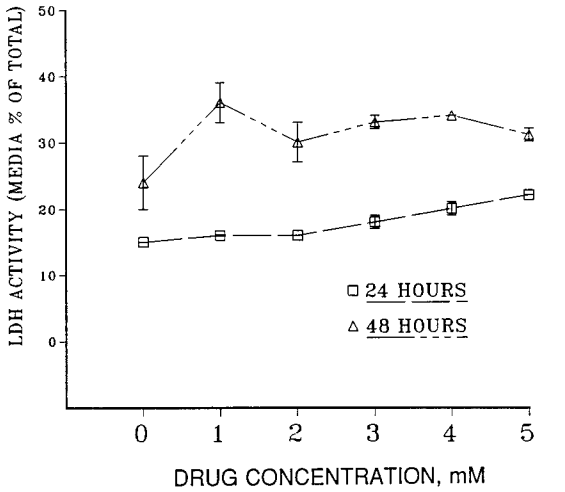


Fig. 6. Cytotoxicity evaluation of the antibiotic, trospectomycin, in cultured human hepatocytes. Cells were isolated from the liver of a 5-year-old male donor, plated on collagen, and after overnight incubation were exposed to drug at concentrations up to 5 mM for 24 or 48 h. Cytotoxicity, as measured by LDH release, was not observed in this or other human isolates. Results are medium LDH values expressed as a percent of the total enzyme activity (medium + cell LDH).

6. Panadiplon: hepatic toxicity observed during clinical development

Panadiplon (Fig. 3), is a non-benzodiazapine anxiolytic that was being developed for treatment of Generalized Anxiety Disorder and Panic Disorder. No evidence of toxicity was observed in preclinical development studies (rat, dog and monkey), but serum transaminase elevations were observed in a few humans during phase I clinical trials. Since no animal model existed for this hepatic toxicity, it was necessary to find one prior to establishing in vitro models. Subsequently, we were able to show that in the rabbit, panadiplon produced a hepatic toxic syndrome that was not produced in other species and that occurred at drug exposure levels relevant to human exposure [26]. The toxicity was detected by an increase in serum transaminases in some animals, and was characterized by hepatic fat deposition and centrilobular necrosis. By conducting mechanistic experiments with cultured rabbit hepatocytes, we were subsequently able to demonstrate that panadiplon inhibited mitochondrial fatty acid β-oxidation and rhodamine 123 transport [27]. This inhibition was also observed in human, but not rat, hepatocytes.

The inhibitory activity of panadiplon on mitochondrial functions increased with exposure time, suggesting a requirement for drug metabolism. Early experiments with a putative metabolite, cyclopropane carboxylic acid (CPCA; Fig. 3), indicated this compound mimicked the inhibitory activities of the parent drug in vitro. When the generation of CPCA in vivo had been confirmed (P.G. Pearson, unpublished observations), subsequent rabbit studies indicated that this metabolite could produce hepatic toxicity identical to panadiplon in rabbits [28]. It was concluded that the cyclopropane group, released as a carboxylic acid metabolite, was responsible for the toxicity observed in rabbits and likely in humans. Interestingly, inhibition of mitochondrial function alone, in vitro or in vivo, was not sufficient to produce hepatocellular death, but rather some form of secondary insult such as hypoxia appeared to be required (manuscript

submitted). Results from these studies were subsequently applied to chemistry synthetic efforts.

7. Conclusion

Cultured hepatocytes are useful models for investigating the toxicity of drugs, including metabolism, biological and chemical mechanisms. Results from in vitro studies, which must be considered in perspective to animal and human data, can provide structure-toxicity information to drug discovery team efforts, explain species differences in hepatic toxicity, and provide an estimate of potential human risk.

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Xenobiotic receptor knockout mice

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Abstract

Administration of certain foreign chemicals to animals elicits responses that are due to receptor-mediated activation of gene expression. Among the most well studied receptors are the Ah receptor (AHR) that binds 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds and the peroxisome proliferator-activated receptors, PPARs, that mediate gene activation by the diverse group of peroxisome proliferators. These receptors may also have critical roles in development or physiological homeostasis in addition to their abilities to allow animals to interact with exogenous chemicals or xenobiotics. To explore the function of AHR and PPARα and to determine whether they participate in the adverse effects of dioxins and peroxisome proliferators, gene knockout mice were developed.

Keywords: Peroxisome proliferators; Dioxin; Ah receptor; Hepatic fibrosis

1. Ah receptor (AHR) knockout mice

1.1. The AHR

The Ah receptor (AHR) is the ligand binding subunit of a dimeric ligand-activated nuclear transcription factor that binds to upstream regulatory elements of target genes [1]. This receptor activates genes after binding to TCDD, polycyclic aromatic hydrocarbons, PCBs and other chemical inducers. In the absence of ligand, the AHR is bound to two molecules of heat shock protein 90 (hsp90). Upon ligand binding, the AHR dimerizes with another protein called Ah receptor nuclear translocator (arnt), coincident with liberation of hsp90. This complex is capable of binding to the Ah receptor regulatory element (AhRE) and activating gene transcription. Both AHR and arnt have similar modular domain structures and are considered members of the helix-loop-helix superfamily of transcription factors that include the *Drosophila* Sim and Per gene products [1]. The AHR is well conserved in mammals and is expressed in early embryos and most tissues and cells of adult animals. To determine the role of AHR in mammalian development and physiology and in the toxic effects of dioxins, the AHR gene was disrupted to produce AHR deficient mice ($AHR^{-/-}$).

1.2. Production of $AHR^{-/-}$ knockout mice

Details for production of $AHR^{-/-}$ have been published [2]. Briefly, a genomic clone containing the first two exons of the AHR gene was isolated from a mouse gene library using the AHR cDNA. Exons 1 and 2 encompass a portion of

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the helix-loop-helix domain of the protein. Following a standard strategy for producing a gene knockout targeting vector, the phosphoribosyltransferase II gene, that confers neomycin and G418 resistance to bacteria and mammalian cells, respectively, was inserted into exon 1 just downstream of the initiator ATG. The herpes simplex virus promoter-driven thymidine kinase expression cassette (HSV-TK) was placed at the 5′ terminal end of the genomic segment and the construct introduced into 129/SV mouse-derived embryonic stem cells (ES) by use of electroporation. Clones growing in the presence of G418 (for positive selection of the phosphoribosyltransferase II gene) and ganciclovir (for negative selection of the HSV-TK gene) were selected. Specific clones were identified by Southern blot analysis using a probe that flanks the targeting vector AHR genomic DNA in the native AHR gene. Three clones, out of 980, were isolated and injected into 3.5-day-old C57BL/6N mouse embryos which, upon development, revealed the presence of 129/SV cells by virtue of the brown coat color on the normally black C57BL/6N mouse background. Mating of these 'chimeric' mice to C57BL/6N revealed germ line transmission of the ES cell genetic background and mice harboring a disrupted AHR allele identified by Southern blot analysis of tail DNA. Heterozygous mice ($AHR^{-/+}$) were bred to yield $AHR^{-/-}$.

1.3. Characteristics of $AHR^{-/-}$ mice

Heterozygous $AHR^{-/+}$ mice appeared normal and were fertile. However, it was clearly evident from the initial $AHR^{-/+}$ breedings, that $AHR^{-/-}$ mice were not normal; from an expected 25% of the homozygous genotype only less than 5% of the pups surviving after 1 week were of the $AHR^{-/-}$ genotype. Most died within 1–4 days after birth. Necropsy of the dead mice revealed the presence of lymphocyte infiltration in various organs, most notably the gut, lung and urinary tract, suggesting that the cause of death was opportunistic infections. $AHR^{-/-}$ mice that survived after the initial crisis period, exhibited a lower growth rate but reached maturity and were fertile. Breeding of $AHR^{-/-}$ mice resulted in small litters of between one and four pups.

The presence of lymphocyte infiltration suggested that the immune system of $AHR^{-/-}$ mice was not normal. Analysis of the spleens revealed smaller than normal periarterial lymphatic sheaths as compared to age-matched normal mice and apparently lower numbers of lymphocytes. The relative ratios of CD4 and CD8 T cells and CD45 B cells were not different between the $AHR^{-/-}$ and $AHR^{-/+}$ mice. Lower lymphocytes were also found in lymph nodes of young $AHR^{-/-}$ animals. The low lymphocyte counts were most prominent in immature and older animals; levels dropped precipitously between 10 weeks and 30 weeks of age. These results suggest that the AHR affects the appearance of peripheral T and B cells.

Other major organs of $AHR^{-/-}$ were examined for histological abnormalities. In young and old mice, the liver exhibited evidence of fibrosis in the periportal area around the bile ducts, hepatic vein and artery. In young animals, 5–10% of the liver had pockets of collagen accumulation, which increased with the age of the animal. In older animals, fibrosis was also found in the lung. The mechanism by which the livers became fibrotic is unknown but probably results from death of liver cells similar to that seen when animals are administered hepatotoxins. Thus, AHR may protect the liver from endogenously-generated toxins or it may be directly involved in liver cell differentiation or apoptosis.

Expression of target genes controlled by the AHR was examined by Northern blot analysis of mRNA levels. Administration of TCDD (40 μg/kg) to mice results in an AHR-mediated transcriptional activation of many genes including *CYP1A1*, *CYP1A2*, and a form of UDP-glucuronosyltransferase *Ugt1*06*. In $AHR^{-/-}$ mice these genes are not induced, thus confirming their requirements for the AHR. *CYP1A2* and *Ugt1*06* are expressed in untreated mice; however, in the knockout mice, this basal expression is lost. These data indicate that AHR is involved in constitutive expression of certain genes by an as yet unknown mechanism.

1.4. Future prospects for the AHR$^{-/-}$ mice

The AHR is clearly required for normal development and physiological vitality in the mouse. The precise mechanism by which it functions in the liver and immune system is unknown and will require further experimentation. The AHR$^{-/-}$ mice will be an important animal model to determine whether the receptor is required for the detrimental developmental, teratologic, toxic and carcinogenic effects of TCDD and polychlorinated biphenyls (PCB). Unfortunately, the mice are too sick for reasonable comparative analysis. In order to attempt to restore health to these animals and investigate some of the deleterious consequences of dioxins, the receptor may have to be expressed in the liver by use of a transgenic approach with a tissue-specific promoter.

2. Peroxisome proliferator-activated receptor α subunit (PPARα) knockout mice

2.1. PPARα

Peroxisome proliferators are a structurally diverse group of chemicals that upon chronic administration to rats and mice cause marked changes in the liver including an increase in peroxisome number accompanied by hypertropy and hyperplasia. Long-term administration of potent peroxisome proliferators results in hepatocarcinogenesis [3]. Genes encoding peroxisomal enzymes, cytochromes P450s in the CYP4A subfamily and mitochondrial enzymes involved in fatty acid metabolism are under control of PPARα. The common linkage of many of the enzymes induced by peroxisome proliferators is their association with fatty acid metabolism, in particular, mitochondrial and peroxisomal B-oxidation. Peroxisome proliferators include the fibrate class of hyperlipidemic drugs such as clofibrate, certain pthalate ester plasticizers, herbicides and some chlorinated hydrocarbons, all of which are chemically unrelated except for a carbon backbone and carboxylate function.

Peroxisome proliferators appear to mediate their effects through a receptor-based mechanism. A family of receptors called peroxisome proliferator-activated receptors (PPAR) were found that can transmit the chemical signal to a transcriptional gene activation response [4]. PPARs share structural modalities with receptors in the ligand-activated steroid, thyroid and retinoic acid receptor superfamily. At least three PPARs are known to exist, PPARα, PPARδ (also called NUC1) and PPARγ, with PPARα displaying properties suggestive of a principal role in the peroxisome proliferator response such as highest level of expression in liver, kidney and heart and the ability to *trans*-activate PPAR response elements (PPRE) in the presence of peroxisome proliferator chemicals [5].

PPARα requires a dimerization partner retinoic acid X receptor alpha (RXRα) and 9-*cis* retinoic acid for full *trans*-activation activity [6,7]. To determine the role of PPARα in the effects of peroxisome proliferators and its possible role in regulation of fatty acid metabolism, the PPARα gene was disrupted to produce mice that lack expression of the receptor.

2.2. Production of PPARα knockout mice

PPARα mice were generated as described earlier [8]. A genomic clone containing exons 7 and 8 of the PPARα gene was isolated from a 129/SV library. These exons correspond to the putative ligand-binding domain of the receptor [9]. The phosphoribosyltransferase II gene was inserted into exon 8 and the HSV-TK gene placed on the 3′ end of the genomic DNA segment prior to electroporation into ES cells. In contrast to production of the AHR$^{-/-}$ mouse, the frequency of specific recombination was 5% of clones selected in the presence of G418 and ganciclovir. Several clones injected gave rise to chimeric mice and germ line transmission of the disrupted allele. Homozygous mice, designated PPAR$\alpha^{-/-}$, were produced and were found to be fertile.

2.3. Characterization of PPAR$\alpha^{-/-}$ mice

Mice having two disrupted alleles appeared to be normal. They reproduced and had large litters of 8–12 pups, in contrast to the low yield of pups made by AHR$^{-/-}$ mice. Older animals are about

20–30% heavier than their normal or heterozygous counterparts. Gross pathological analysis revealed normal organ structures. Histological examinations uncovered a considerable accumulation of fat droplets in the liver and kidney. Withdrawal of food for 2 days resulted in marked hepatohypertropy and a marked increase in liver fat deposits in the PPAR$\alpha^{-/-}$ mice. No increase in fat deposits were found in normal mice that were starved for a similar period of time.

Administration of the peroxisome proliferators clofibrate (0.5% wt/wt) or WY-14,463 (0.1% wt/wt) for 2 weeks did not result in an increase in cellular levels of peroxisomes in the PPAR$\alpha^{-/-}$ mice, whereas the same treatment caused a typical pleiotropic response in either normal or PPAR$\alpha^{-/+}$ mice. Livers of PPAR$\alpha^{-/-}$ mice were the same weight in the treated and untreated groups while normal mice exhibited a 100% increase in liver weight upon administration of WY-14,463. Analysis of the livers of treated PPAR$\alpha^{-/-}$ mice for mRNAs encoding target genes revealed no evidence for induction of genes encoding two peroxisomal enzymes, two microsomal CYP4A cytochromes P450 and the liver fatty acid binding protein. A slight increase in thiolase was noted. In contrast, and as expected, induction of enzymes in normal mice was highly responsive to both clofibrate and WY-14,463.

2.4. Future prospects for the PPAR$\alpha^{-/-}$ mice

Studies to date on the PPAR$\alpha^{-/-}$ mice indicate that they are resistant to the pleiotropic effects of peroxisome proliferators demonstrating the role of the receptor in this process. These studies are in support of earlier work showing that PPARα is the only member of the PPAR family capable of eliciting a *trans*-activation response with WY-14,463 [5]. The marked accumulation of fat in the liver suggest that PPARα is also responsible for regulation of fatty acid catabolism in the liver. Indeed, metabolites of long chain fatty acids, possibly the dicarboxylic acids, can stimulate the peroxisome proliferation response [10,11].

2.5. Marked species differences in response to peroxisome proliferators

Rats and mice are highly responsive and are susceptible to hepatocellular carcinomas after chronic administration of peroxisome proliferators. Humans and other primates are thought to be weakly responsive or nonresponsive. These species differences have implications for the pharmaceutical industry and regulatory agencies since hyperlipidemic drugs are on the market and other compounds are under development that stimulate peroxisome proliferation-like activities. The mechanisms by which humans are resistant to the effects of peroxisome proliferators are not presently understood but recent studies suggest that low hepatic levels of PPARα may be in part responsible (Eric F. Johnson, personal communication). Studies are planned to determine whether PPAR$\alpha^{-/-}$ mice are susceptible to the carcinogenic effects of WY-14,463 [3].

3. Conclusion

Gene targeted disruptions have revealed that the AHR and PPARα have endogenous roles in normal development and homeostasis. AHR affects the liver and immune system and PPARα alters hepatic fatty acid catabolism. Future studies should reveal whether these receptors are involved in the toxic and carcinogenic properties of dioxins and peroxisome proliferators.

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Transgenic approaches to understanding the mechanisms of chemical carcinogenesis in mouse skin

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Abstract

The use of animal models for human cancer has proved effective in the elucidation of those molecular events which are responsible for the various stages of tumour development. Chemical carcinogenesis in mouse skin has been studied as a model for human squamous cancer for several decades, and analysis of this model has led to the identification of a number of the changes which are involved in the evolution of malignancy. The use of transgenic and knockout mice offers a further avenue of advancement, allowing refinement of the model, and the ability to examine the consequences of individual events in vivo in greater detail. Additionally, crossing different transgenic or knockout animals represents a powerful tool to study the cumulative effects of several genetic alterations acting in concert.

Keywords: Papilloma; Squamous carcinoma; H-*ras* oncogene; p53 Tumour suppressor gene; Malignant conversion; Tumour stem cell

1. Introduction

Carcinogenesis in humans and in animals is a multistep process, with multiple genetic events required to confer upon a cell a neoplastic phenotype. These changes act at a different stage of tumour development to endow a selective growth advantage to that cell, and thus precipitate advancement to the next stage of the process. Each genetic alteration may provide a positive growth stimulus to the cell and thus lead to increased cell proliferation, or may result in the disruption of negative regulatory mechanisms or alteration in the rate of cell loss through terminal differentiation or apoptosis. In each case the end product is a cell which is capable of clonal expansion, leading in turn to the next stage in the evolution of malignancy. The genes which are involved in these types of somatic mutation have been broadly categorised into 2 classes, oncogenes and tumour suppressor genes. Members of the oncogene class are genes which become functionally activated by mutation, and whose expression now leads to altered cell behaviour. Since the mutational alteration activates the gene, it need occur in only 1 of the 2 alleles present in the genome. The second group, the tumour suppressor genes become functionally inactivated by mutation. It is this loss of function

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which then results in the altered properties of the tumour cell. Since 2 copies of a tumour suppressor gene are normally present in the genome, both copies must be inactivated for the effect to be unmasked. Indeed, this type of gene had been predicted by epidemiological studies on inherited and sporadic childhood cancers which suggested a 2-hit mechanism at a single genetic locus [1]. The inheritance of an inactivated suppressor allele is responsible for many human familial tumour syndromes, such as familial retinoblastoma, in which affected individuals carry germline mutations in the retinoblastoma (Rb) tumour suppressor gene [2]. Many other examples are now known of suppressor genes which are responsible for inherited tumour predisposition [3,4].

In addition to the advances in the molecular biology of neoplasia, the development of transgenic mice has occurred in which exogenous fragments of DNA can be introduced into the mouse genome. This has provided a powerful in vivo approach by which the roles of individual genes involved in neoplasia can be examined. Transgenic mice can thus be used to investigate the roles of oncogenes in tumorigenesis, by introducing the genes under their own promoters or by targeting expression to particular cell populations using tissue-specific promoters [5]. In contrast, gene targeting in embryonic stem cells can be used to produce mice which have knocked out a specific tumour suppressor gene [6]. Our studies, centered on mouse skin carcinogenesis, have been concerned with characterising the genetic events which are responsible for the development of neoplasia, and investigating the biological consequences of these alterations in somatic cells. A major advantage of mouse model systems is that the causal role of changes found in tumours can be tested by using transgenic or knockout mice.

2. Results and discussion

Chemical carcinogenesis in mouse skin represents a classic model for multistage carcinogenesis, and has been extensively studied [7]. From the observations that tumour formation proceeded through distinct stages has arisen the concept of multistage carcinogenesis, and the operational definitions of initiation, promotion and progression which have been widely applied to the development of neoplasia in humans. More recent studies carried out over the last decade have identified a number of genetic alterations which occur during these stages of skin tumour development (Fig. 1). Frequently, initiation with the carcinogen dimethylbenzanthracene (DMBA) produces a specific mutation in codon 61 of the Harvey *ras* gene (H-*ras*) [8]. These initiated cells can be induced to develop into benign papillomas by subsequent application of a tumour-promoting agent such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Two distinct groups of papilloma appear to be produced, one group which shows a low risk of progression to malignancy, and a second group which show a much higher frequency of malignant progression. Tumours belonging to the latter category progress to form squamous carcinomas which invade

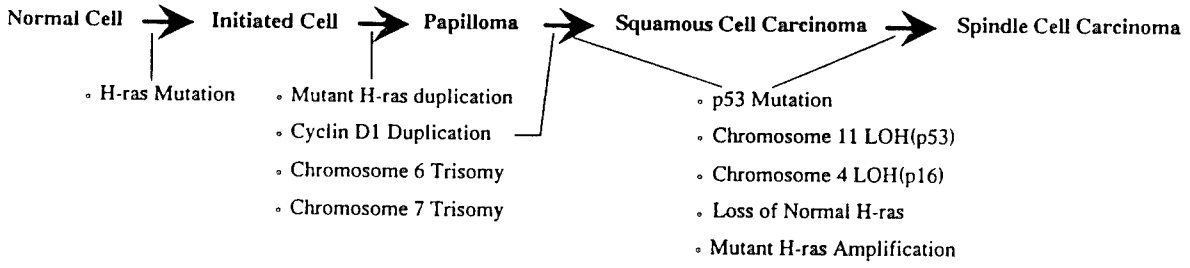


Fig. 1. Summary of genetic alterations observed during mouse skin carcinogenesis. Mouse skin initiated with a single dose of DMBA and promoted for 20 weeks with TPA gives rise to papillomas, a small percentage of which convert to squamous carcinomas. Genetic changes were detected by mutation analysis and allelotype analysis of tumour DNA.

the dermis. Loss of heterozygosity (LOH) on chromosome 11 and mutation of the p53 suppressor gene have been demonstrated to be involved at this stage of tumorigenesis [9]. Several other genetic changes have been associated with papilloma formation, namely trisomies of chromosome 7, carrying the H-*ras* gene, and chromosome 6. Trisomy of chromosome 7 invariably involves duplication of the chromosome containing the mutant H-*ras* allele, and from the analysis of a large number of tumours, duplication of the normal H-*ras* allele has never been observed. In addition, whilst some tumours which contain mutant H-*ras* show no evidence of chromosome 7 trisomy, it is never detected in those tumours which lack mutant H-*ras*. Thus trisomy of 7 in a cell probably serves to increase the level of mutant H-*ras* expression, leading to selective outgrowth from the normal population. In support of this proposal, trisomy of 7 is not seen in tumours from animals with a mutant *ras* transgene. The reason for trisomy of chromosome 6 has not yet been determined. A likely explanation, however, involves a selective growth advantage conferred by the duplication of a gene or genes, of which there are several possible candidates, including transforming growth factor α (TGF-α), K*ras*, and c-*raf*-1. The c-*raf*-1 protein kinase is of particular interest, as it lies downstream from *ras* in the signal transduction pathway from growth factor receptors to transcriptional activation in the nucleus. *raf* has been shown to be present at limiting concentrations in some cell types and an increase in the amount of *raf* can potentiate the effects of stimulating the *ras* signal transduction pathway [10]. Thus the duplication of c-*raf*-1 chromosome 6 in mouse skin tumours may be required to synergise with the increased levels of H-*ras* and lead to amplification of growth stimulatory signals via signal transduction pathways involving *ras* and *raf*.

A further stage of tumour progression is the conversion of a squamous carcinoma to a spindle cell carcinoma, which lacks all characteristics of epithelial differentiation. This stage is associated with additional genetic changes, including LOH on chromosomes 6, 7, and 4. Significantly, these regions are syntenic with the human 3p, 11p15, and 9q regions respectively, which show frequent LOH in various human tumours and are likely to contain tumour suppressor genes.

2.1. Tumour progression and p53

Mutation of the p53 tumour suppressor gene is the most frequent single genetic lesion detected in human tumours. Additionally, the mutation is found more frequently in the more malignant late stages of tumour development than in early stages, suggesting that loss of function of p53 is important in malignant progression [11]. A similar situation is observed in mouse skin tumours. Mutation of p53 and LOH on chromosome 11 were found in approximately 25% of carcinomas, though were never detected in benign papillomas [9]. Previously we had shown that mutations induced in the H-*ras* gene were dependent upon the initiating carcinogen used, supporting a causal role for H-*ras* mutation in the initiation step of carcinogenesis [12]. Similarly, mutations detected in the p53 gene were dependent upon the treatment regimen used (Fig. 2, P.A. Burns et al., unpublished). Tumours induced by initiation with DMBA and TPA promotion exhibited typical loss of function mutations in p53, such as frameshift mutations, small deletion mutations and point mutations introducing stop codons. In addition, the remaining wild-type allele was lost. In contrast, carcinomas induced by repeated carcinogen treatment using DMBA or the

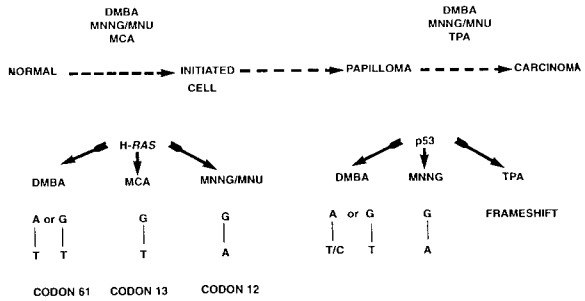


Fig. 2. Carcinogen-specific changes in H-*ras* and p53 genes. Papillomas and carcinomas initiated with different carcinogens exhibit specific mutations in the H-*ras* proto-oncogene (12). Similarly, carcinomas promoted or progressed with different carcinogens show specific mutations in the p53 gene [9] and P.A. Burns, unpublished results.

methylating agent 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), showed carcinogen-specific alterations in p53. Of 5 p53 mutations detected in DMBA-induced carcinomas, 1 deletion and 4 transversions were found. Mutations were found in 4 carcinomas induced by multiple MNNG treatment and in each case these were G → A transitions. Loss of the remaining wild-type p53 allele was not observed in these tumours. These results support a causal role for p53 in the progression from benign to malignant tumour. This hypothesis was further confirmed by studying skin carcinogenesis using p53-deficient mice. Groups of wild-type, heterozygous $p53^{+/-}$, and homozygous p53 null mice were initiated with DMBA and promoted with TPA for 15 weeks [13]. Papilloma formation in $p53^{+/-}$ cohort was very similar to that for wild-type animals, showing that a 50% reduction in the dosage of normal p53 gene does not affect the number or growth rate of benign papillomas. Surprisingly, the papilloma yield in null mice was markedly reduced, possibly due to the increased sensitivity of these keratinocytes to the effects of DMBA and TPA. No tumours were observed in null mice treated with TPA alone, demonstrating that inactivation of p53 cannot function as an initiating event.

A marked effect upon malignant conversion was apparent, however, in both the heterozygous and null cohorts. p53 heterozygous mice exhibited a 3-fold increase in the progression frequency of papillomas to carcinomas. Correspondingly, loss of the remaining wild-type allele was associated with this conversion. In the null animals, carcinoma development was dramatically accelerated, with the first carcinomas appearing after 10–12 weeks, less than half the latency period observed in the $p53^{+/-}$ cohort. Additionally, carcinomas from the null mice were less differentiated and more malignant than those from wild-type mice, with a higher frequency of metastasis. These experiments clearly support a functional role for wild-type p53 in the prevention of tumour progression. Cells within the benign tumour which lose p53 function may then be selected due to their escape from p53-mediated growth arrest or apoptosis.

2.2. Initiation and the target cell for carcinogenesis

One unresolved question of squamous skin tumours is in the nature of the initiated cell from which the tumour evolves. Since both high and low risk papillomas contain the same mutant H-*ras* initiating event, their different malignant capacities may be due to their having arisen from different tumour stem cells. The use of promoter elements from various cytokeratin genes offers a unique opportunity to examine the consequences of expressing genes, which are implicated in the initiation or progression stages of carcinogenesis, to particular subpopulations of cells within the epidermis (Fig. 3). We have previously targeted the expression of a mutant H-*ras* gene in the suprabasal differentiating cells of the epidermis using the promoter of the keratin 10 gene [14]. The transgenic mice that were produced exhibited extensive epidermal hyperkeratosis over much of the body surface. Mild mechanical irritation such as wounding or scratching resulted in the focal induction of hyperplasia which culminated in the production of papillomas at these sites. Thus H-*ras* expression would appear to control growth and differentiation in these cells. Significantly, during the course of these experiments, we saw no evidence of malignant progression in these papillomas. Similar results have been demonstrated subsequently by Greenhalgh et al., who have shown that expression of v-*ras* from a keratin 1 promoter gives rise to benign papillomas which do not progress to malignancy [15]. Both these situations, therefore, reproduce the early stages of tumour development in mouse skin, but give rise specifically to the low risk or terminally benign papilloma type.

More recently, we have attempted to induce benign tumours belonging to the high risk group by targeting a mutant H-*ras* gene to an alternative cell population within the epidermis. The keratin 5 gene promoter is normally functional in the basal cells of all stratified epithelium, however using a shortened promoter fragment, we have restricted expression from the promoter mainly to the outer root sheath of the hair follicle. Using this promoter to express mutant

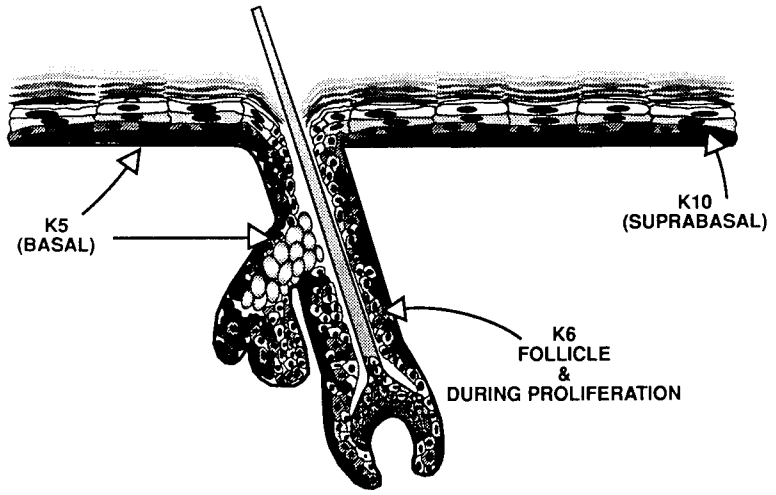


Fig. 3. The use of cytokeratin promoters allows expression of transgenes to be targeted to different populations of cells within the epidermis. The K10 promoter directs expression to the suprabasal differentiating cells, whereas the K5 promoter functions in basal interfollicular cells together with outer root sheath cells of the hair follicle. The K6 promoter is usually restricted to the hair follicle, though functions more widely during hyperproliferation.

H-*ras* (K5*ras*KT) resulted in a number of founder animals which developed acanthotic skin lesions arising from abnormal follicle growth, either during development or shortly after birth. The acanthotic lesions frequently exhibited areas of carcinoma in situ and invasive carcinoma. Approximately 20–30% of adult animals, from several established lines, spontaneously developed papillomas and keratoacanthomas, many of which progressed to squamous, and occasionally, spindle carcinomas. These particular mice are therefore capable of reproducing the complete process of tumorigenesis in mouse skin. The actual tumour incidence varied depending upon the strain background, with strain FVB/N showing the highest tumour incidence and strain C57Bl/6J the lowest of those examined. Surprisingly, tumours could not be induced in the mice by promotional stimuli, such as wounding or phorbol ester treatment. The mice also develop occasional tumours in other tissues, most frequently sebaceous adenomas and zymbal gland tumours, salivary papillomas from the epithelial ducts of the salivary glands in approximately 15% of animals, and mammary carcinomas in female breeders (5%).

The experiments described above demonstrate that the target cell for H-*ras* gene expression strongly influences the phenotype of the resulting tumour (Fig. 4). Expression of mutant *ras* in cells that have advanced some way along the differentiation pathway in skin, i.e. have reached the interfollicular or even suprabasal compartment of the epidermis, effects excessive differentiation and with promotional stimulus can induce only benign papillomas. Expression of the same gene in the outer root sheath of the hair follicle, where the putative stem cell population resides [16], is characterised by excessive cell proliferation and the autonomous formation of benign tumours, which have a high probability of progression. In conclusion, these results support the hypothesis that the difference in progression potential between high and low risk papillomas, which is observed in chemical carcinogenesis, may be a consequence of initiation occurring in different cell populations within the skin.

2.3. TGF-α and ras in skin carcinogenesis

TGF-α is a major autocrine factor controlling growth in epidermal cells, and elevated levels of TGF-α have been detected in squamous tumours

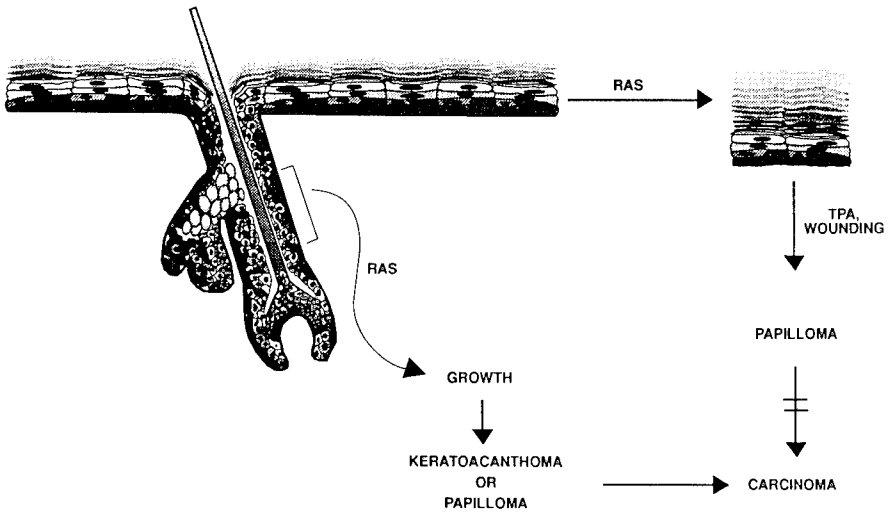


Fig. 4. The expression of mutant *ras* in suprabasal cells affects differentiation and upon promotion results in low risk or terminally benign papillomas. *Ras* expression in the hair follicle results in proliferation and gives papillomas or keratoacanthomas which have a high risk of malignant conversion.

from human and mouse. Introduction of a mutant H-*ras* gene has been shown to upregulate the production of TGF-α in keratinocytes, as has treatment with TPA. Several groups have used transgenic mice to examine the role of TGF-α in skin carcinogenesis [17,18]. Both studies reported similar findings, with mice exhibiting hyperkeratosis and hyperplasia, consistent with TGF-α affecting keratinocyte proliferation. Moreover, papillomas occurred at sites of wounding or mechanical irritation, and could be promoted by TPA treatment. None of the mice, however, developed malignant squamous carcinomas. Thus TGF-α overexpression is able to reproduce many of the effects of mutant H-*ras*, and to function as an initiating event in mouse skin, at least for benign tumours. Some of the effects of mutant *ras* may, therefore, be due to TGF-α overexpression.

We have utilised mice which are deficient in the gene for TGF-α [19] in order to examine its role in skin carcinogenesis. Crossing TGF-α knockout mice with K5*ras*KT mice allowed the generation of mice which contain mutant *ras* but which lack TGF-α. Our results show no difference in the frequency of spontaneous benign and malignant tumour formation between the mutant *ras* mice in either TGF-α null, heterozygous or wild-type background. Thus the lack of TGF-α does not appear to inhibit the development of high risk papillomas from initiated cells within the hair follicle. In view of this result, we have examined the response of TGF-α null mice in 2-stage chemical carcinogenesis. Groups of TGF-α null and wild-type mice were initiated with DMBA and promoted with TPA for 20 weeks. Papillomas in the TGF-α null cohort were greatly reduced in number and showed an increased latency, with the first tumours occurring after 18 weeks, compared to 8 weeks in wild-type animals. The carcinoma incidence, however, did not appear to be similarly affected, with the first carcinoma occurring at week 30. Thus TGF-α may be required for the development of low risk papillomas which arise by a proliferative mechanism in the interfollicular epidermis, and this type of papilloma cannot be induced in TGF-α null mice. Further experiments are in progress to validate this hypothesis.

2.4. Hair follicle tumour stem cells and p53

Since p53 deficiency in mice has been found to significantly affect malignant conversion in carcinogen-treated mice, we subsequently examined

the effect of p53 inactivation upon tumour development in our K5*ras*KT transgenic mice. K5*ras*KT mice were bred with p53 null mice to produce mutant *ras* transgenics with p53 wild-type, $p53^{+/-}$, and p53 null genotypes. All 3 groups showed similar incidences of spontaneous skin tumours, though results for the p53 null group were affected by loss of animals due to lymphoma and sarcoma development. Thus, p53 status does not appear to influence the yield or malignant conversion of papillomas or keratoacanthomas which develop from the hair follicle. A similar situation has been reported for liver tumours induced by diethylnitrosamine, in which p53 heterozygous mice show neither an increase in the number nor the progression frequency of liver tumours over that found in wild-type animals [20].

There was, however, a significant effect upon salivary tumorigenesis. Wild-type animals developed hyperplasia and papillomas in the epithelial ducts, however malignant tumours were never observed within the lifetime of the animals. In contrast, p53 heterozygous animals developed salivary tumours in higher numbers and these were frequently malignant carcinomas. Clearly a marked difference exists regarding the role of p53 in the progression of skin and salivary papillomas. This may reflect differences in the initiated cells between these 2 organs. The salivary papillomas may arise from a cell resembling an interfollicular-initiated cell in the skin, i.e. a low risk group which requires p53 mutation to convert to malignancy. Further studies are in progress to examine this proposal.

3. Conclusions

Studies of carcinogenesis have been greatly enhanced by the application of transgenic mice. The causal nature of many of the genetic alterations in oncogenes and tumour suppressor genes which are observed in tumour development can be clearly demonstrated. In addition, the targeting of these mutations to different sub-populations of cells within a particular tissue allows a degree of experimental manipulation which would be difficult to accomplish by other means. Similarly, studies on the synergism between mutations are easily achieved by crossing individual transgenic mice. Experiments such as those described above demonstrate the unique advantages offered by transgenic mice in appraising our understanding of neoplasia.

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Toxicology Letters

Transgenic models for detection of mutations in tumors and normal tissues of rodents

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Abstract

Transgenic rodents that contain easily retrievable target genes allow the rapid quantitation of mutations in any tissue from which DNA can be isolated. We are using the Stratagene Big Blue™ transgenic mouse system that contains a *lacI* target and an *αlacZ* reporter gene to study the parameters that affect mutations. We have evaluated a number of chemicals to determine mutant frequency (MF) in specific target tissues of C57Bl/6 and B6C3F1 mice. The correlation between mutagenesis and carcinogenesis in this system is excellent. For example, the liver carcinogen dimethylnitrosamine produces significant increases in MF in mouse liver, whereas the nonhepatocarcinogenic mutagen methylmethane sulfonate does not. We have also evaluated the induction of mutations by radiation and demonstrated that this system is suitable for the study of agents that produce deletion mutations. This system is also useful for studying changes in MF in developing tumors. We have used an initiation-promotion protocol to induce hepatocellular carcinomas, and we then measured MF in normal liver, tumors, and metastases from these mice. Animals initiated with diethylnitrosamine maintain an elevated MF in normal liver, even 1 year after initiation. This MF increases exponentially in developing liver tumors, possibly owing to a breakdown in the fidelity of DNA replication and DNA repair in tumors. This system offers a unique tool for the study of mutations induced in specific target tissues of rodents and should become an important assay for evaluating the mutagenic risk of drugs and chemicals.

Keywords: Transgenic animal; Mutagenesis; In vivo mutations; Cancer; Genotoxicity

Birth defects, cancer, and other diseases have been shown to occur as a result of mutations in specific genes. Indeed, cancer has been shown to be the result of a series of mutations in specific oncogenes and tumor suppressor genes [1]. Until recently, the only models available for the study of chemically induced mutations were in vitro mutagenesis assays or in vivo assays that measured surrogate end points such as chromosome damage or DNA repair.

A revolutionary advance in this field has been the development of transgenic animals carrying a specific target gene that allows easy quantitation of mutations [2,3]. The commercially available transgenic mutagenesis systems use the *lacI* or *lacZ* target genes from the bacterial β-galactosidase operon as a target. Our laboratory has used the Big Blue™ system, marketed by Stratagene (La Jolla, CA), which contains a *lacI* target gene and a *lacZ* reporter gene. This model is available in B6C3F1 and C57Bl/6 mice [4], and development of a F-344 rat model is under way [5]. The *lacI* gene is the most characterized of all target genes for mutagenesis. To date, more than

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30 000 mutants from both prokaryotic and eukaryotic hosts have been sequenced [6–8]. In this system, rats or mice are treated with chemicals, and after a sufficient time to allow fixation of DNA adducts as mutations, genomic DNA is isolated; finally, the target gene is recovered by exposing the DNA to λ-phage in vitro packaging extracts and infecting *Escherichia coli* with the λ-phage. Successful infection of the *E. coli* results in lysis of cells and formation of plaques on the bacterial lawn. Infection with a normal *lacI* gene results in production of a repressor molecule that inhibits transcription of the *lacZ* gene, which codes for β-galactosidase. If a mutation has been produced in *lacI*, however, the repressor is not produced, and *lacZ* is transcribed, resulting in the presence of β-galactosidase. β-Galactosidase activity can be measured in *E. coli* by plating on media containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Presence of β-galactosidase activity results in a blue colony or plaque, whereas absence of activity results in a white colony or clear plaque. Mutant frequency (MF) is determined as the ratio of blue plaques (*lacI* mutants) to clear plaques.

Our laboratory became interested in evaluating transgenic models as a means to assess tissue-specific mutations. An early study conducted in our laboratory [9] evaluated the effects of 2 methylating agents, dimethylnitrosamine (DMN) and methylmethane sulfonate (MMS). These 2 compounds were selected as representative mutagens that form methylated DNA adducts. Both DMN and MMS are in vitro mutagens that produce a variety of DNA adducts consisting primarily of N^7-methylguanine; however, there are 3 principal differences between these agents: (1) DMN also produces the highly mutagenic O^6-methylguanine adduct, whereas MMS does not; (2) DMN produces significant hepatic cell turnover, whereas MMS does not; and (3) DMN is a potent liver carcinogen, whereas MMS is not. We demonstrated that DMN is a potent mutagen in the livers of *lacI* transgenic mice, whereas MMS is not (Fig. 1), and we attribute this response to differences in the spectrum of adducts formed and to the absence of MMS-induced cell proliferation [9].

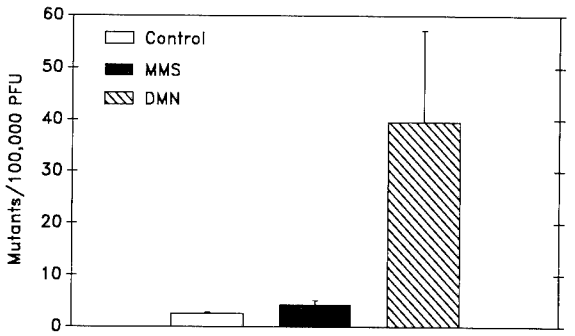


Fig. 1. Induction of mutations, expressed as number of mutants per 100 000 plaque forming units (PFU), in male B6C3F1 mice by MMS (20 mg/kg) or DMN (2 mg/kg). Compounds were given in saline by i.p. injection for 21 consecutive days followed by a 1-day expression period. Controls received saline injections. (Adapted from Ref. [9]).

Induction of cell proliferation is also a prerequisite for DMN-induced mutations. At doses (2 mg/kg/day) that failed to induce significant hepatotoxicity or cell turnover, DMN did not produce mutations. Increasing the dose to 4 mg/kg/day crossed the toxicity threshold and a significant increase in hepatotoxicity occurs at this dose, and significant elevations in cell replication result. At this dose, DMN also produces a significant increase in the MF. Therefore, at doses in which cell proliferation is induced, DMN produces mutations, but in the absence of cell proliferation, DMN fails to induce hepatic mutations.

The results of this study demonstrate that transgenic systems allow us to answer fundamental questions about the mechanisms of mutation, but they have also raised questions about the definition of the terms 'genotoxic' and 'nongenotoxic'. MMS induces significant DNA adducts and unscheduled DNA synthesis (UDS) in mouse liver and would therefore clearly be classified as 'genotoxic' in the liver. Nevertheless, MMS fails to induce *lacI* mutations in B6C3F1 mouse liver, even when administered for up to 21 days, and would therefore be classified as 'non-

mutagenic' in mouse liver. The value of the transgenic mouse assay is its ability to evaluate *relevant* genotoxicity in multiple target tissues (i.e., heritable mutations). In fact, although MMS is 'genotoxic' in mouse liver, it does not produce liver tumors. Therefore, DNA adduct or UDS assays may be better predictors of 'genotoxicity', but a transgenic mouse model may be a better predictor of mutations and cancer.

One potential limitation of these transgenic mutation systems is that the *lacI* and *lacZ* target genes are relatively small (1–3 kb) and require intact surrounding *cos* restriction sites for successful packaging into the phage head. Therefore, large-scale deletions, such as those produced by ionizing radiations are theoretically unlikely to be detected using these systems, because any deletion that removed either *cos* site would prevent packaging of the *lacI* gene. We have conducted studies to evaluate the ability of the *lacI* system to detect deletion mutations [10]. A significant increase in MF was observed following irradiation of C57Bl/6 *lacI* mice with gamma rays, and approximately 20% of the mutations were identified as deletions by restriction digest analysis. Subsequent sequencing revealed these deletions to range in size from 45 to 249 bp. These results are encouraging because they suggest that some deletions may indeed be detected with this system; however, it is highly likely that a significant proportion of deletions will not be detected, and these models should therefore be used with caution when one is evaluating agents known to cause large-scale deletions.

Another area of interest in our laboratory has been the induction of mutations by nonmutagenic chemicals. Although nonmutagenic chemicals, by definition, do not directly induce DNA alterations, the widely accepted multi-step model of carcinogenesis [1] requires that tumors induced by nongenotoxic carcinogens must be due to an increase in the number of mutant cells by means other than direct interaction with DNA. We have conducted several studies to evaluate the mutagenic potential of these 'nonmutagenic' compounds.

In one experiment we treated male B6C3F1 *lacI* mice with 5 daily doses of carbon tetrachloride (CCl_4, 35 mg/kg/day), phenobarbital (PB, 100 mg/kg/day), or DMN (6 mg/kg/day) and sacrificed animals 7 days after the first dose. Mice were implanted with a 7-day Alzet osmotic pump loaded with [^{3}H]thymidine at study initiation to measure the labeling index (LI; percent of hepatocytes in S-phase) throughout the entire duration of the experiment. Controls had a LI of 0.07% and a MF of $\leq 6 \times 10^{-5}$. DMN produced severe necrosis, and only modest regeneration occurred (LI = 0.34%, a 5-fold elevation over control levels) within the short time period posttreatment; however, DMN treatment still yielded a 5-fold increase in MF. In contrast, CCl_4 and PB yielded highly significant increases in LI. In particular, CCl_4 produced nearly a 1000-fold elevation in LI. If cell proliferation alone resulted in mutations, CCl_4 and PB would be expected to produce significant increases in the MF; however, there was no increase in the MF following treatment with either compound [2,11].

These results indicate that relatively short-term bursts of cell proliferation alone do not produce mutations in the liver; however, long-term administration of nongenotoxic cell proliferators could presumably result in clonal expansion of spontaneous mutations or in selective expansion of populations with mutations in specific oncogenes, and this expansion may be the principal mechanism of carcinogenesis by nongenotoxic chemicals.

To test this hypothesis, we have evaluated mutations in chemically induced liver tumors in *lacI* B6C3F1 mice [2,12]. Unlike oncogenes or tumor suppressor genes such as *ras* or *p53*, the *lacI* gene confers no selective growth advantage or disadvantage to a mammalian cell by its presence or absence; therefore, it is useful as a physiologically neutral marker of mutation rates in tumors. We initiated mice with diethylnitrosamine (DEN), then promoted with the nongenotoxic liver tumor promoters WY-14,643 (WY) or PB. Animals were necropsied after approximately 6 or 12 months. All mice receiving DEN + PB or DEN + WY had multiple liver tumors, whereas mice receiving untreated feed after DEN initiation had few tumors. Nontumor

tissue from mice treated with DEN yielded MF approximately 10-fold higher than control MF at both 6 and 12 months. A tumor arising from a cell that contained a *lacI* mutation would be expected to have a MF of 1/40 (2.5%), assuming only 1 of the 40 copies of the *lacI* gene carried a mutation. Tumors arising from cells that did not contain a *lacI* mutation would be expected to have a very low MF, similar to that seen in controls, and probably lower than the 10-fold increase in MF observed in DEN-initiated (non-tumor) liver.

We found that nearly all tumors analyzed had a MF much higher than in controls, and even much higher than in DEN-initiated normal liver. Preliminary DNA sequencing of these mutants indicates that these are all unique mutations and not the result of clonal expansion. These results indicate that the increase in mutation rate in tumors cannot be explained by clonal expansion. Other factors, such as decreases in fidelity of DNA repair or replication, or the activation of 'mutator' genes that enhance mutation rates, may be responsible for the genomic instability, and subsequent increase in MF observed in tumors.

The studies described above demonstrate the utility of transgenic animal mutation models for assessing tissue-specific genetic risk and for elucidating the mechanisms of chemical carcinogenesis. These assays can fill an important gap in the currently available technologies for assessing genetic risk.

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Toxicology Letters

DNA degradation and proteolysis in thymocyte apoptosis

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Abstract

Data from a number of model systems support a role for proteolysis in apoptotic cell death. Using immature rat thymocytes, we demonstrate that the protease inhibitors *N*-α-tosyl-L-lysinyl-chloromethylketone (TLCK) and benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethylketone (Z-VAD.FMK) inhibit apoptosis. *N*-tosyl-L-phenylalaninyl-chloromethylketone (TPCK) has a very different effect, inducing the early morphological and biochemical changes associated with apoptosis. TLCK inhibits trypsin-like proteases whilst Z-VAD.FMK inhibits interleukin-1β-converting enzyme (ICE)-like proteases; this and the contrasting effects of TPCK support the hypothesis that thymocyte apoptosis involves a hierarchy of proteases which act at different stages of the process.

Keywords: Thymocyte apoptosis; TLCK; Proteases; Interleukin-1β

1. Introduction

Cell death is an irreversible loss of structure and function. Cells die primarily by one of two major mechanisms, i.e. necrosis or apoptosis [1]. Cell death by necrosis occurs as a result of a marked toxic or physical insult. Apoptosis is a process in which individual cells die in a controlled manner in response to specific stimuli following an intrinsic programme [1]. Apoptosis plays a key role in the control of cellular populations in development, in the immune system and in carcinogenesis [1,2]. In vivo, apoptosis characteristically affects individual cells and is not accompanied by an inflammatory reaction. Morphological changes during apoptosis are characterised by condensation of chromatin, nucleolar disintegration, decrease in cell volume and dilatation of the endoplasmic reticulum [1,2]. Following the early morphological changes, fragmentation of the cell into apoptotic bodies often occurs. Apoptotic cells and bodies are rapidly phagocytosed, so that their half-life in vivo is very short.

The most distinctive biochemical characteristic of apoptosis is the 'DNA ladder' observed on agarose gel electrophoresis. This arises from the internucleosomal cleavage of DNA resulting in nucleosomal fragments of 180–200 bp and multiples thereof [3]. The origin of the DNA ladder most probably arises from activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease, which cleaves DNA at internucleosomal regions. The endonuclease may be NUC-18, DNAase I or DNAase II (reviewed in [4]). Recently work from our laboratory and others have suggested that the DNA

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ladders are derived from large kilobase pair fragments of DNA [4,5].

Protein degradation has also been implicated in apoptosis in both invertebrates and mammals. In the nematode, *Caenorhabditis elegans*, the gene *ced-3* is essential for apoptosis and encodes a protein similar to the mammalian protease, interleukin-1β-converting enzyme (ICE) [6]. In rat fibroblasts, over-expression of *ced-3*, ICE or the related *nedd-2* results in apoptosis [6–8]. In thymocytes, inhibitors of proteolysis prevent internucleosomal DNA cleavage [9] and a role for serine and cysteine proteases has been proposed [10,11].

The data presented here are an extension of our previous work on the role of proteolysis and DNA degradation in the sequence of events leading to apoptosis in thymocytes [12]. Apoptosis was induced by agents acting by different mechanisms, i.e. dexamethasone, a glucocorticoid, staurosporine, a protein kinase inhibitor and γ-irradiation. *N*-α-tosyl-L-lysinyl-chloromethylketone (TLCK), *N*-tosyl-L-phenylalaninyl-chloromethylketone (TPCK), and ICE-like protease inhibitors have been used in this study. Both TLCK and TPCK inhibit some cysteine and serine proteases [13]. However, TLCK irreversibly inhibits trypsin-like proteases, which require a basic amino acid in the P1 position (Schecter and Berger nomenclature), while TPCK irreversibly inhibits chymotrypsin-like proteases, which require an aromatic amino acid in the P1 position [13]. In contrast ICE-like protease inhibitors require an aspartate in the P1 position [8,14].

2. Materials and methods

All media and sera were from Gibco (Paisley, UK). Pronase, TLCK and TPCK were from Boehringer Mannheim (Mannheim, Germany). An ICE-like protease inhibitor, benzyloxycarbonyl-valinyl-alaninyl-aspartyl (OMe) fluoromethylketone (Z-VAD.FMK) was from Enzyme Systems Inc. (Dublin, CA, USA). All other chemicals were from Sigma Chemical Company (Poole, UK).

2.1. Preparation and incubation of thymocyte suspensions

Suspensions of thymocytes from immature male F344 rats (4–5 weeks old, 65–85 g) were prepared and incubated as described previously [12]. Populations of predominantly proliferating and quiescent thymocytes (F1 and F2 respectively) were sorted as previously described [15].

2.2. Apoptosis assessed by flow cytometry

Thymocytes (1×10^6) were stained with Hoechst 33342 and propidium iodide and viable apoptotic and normal cells separated by flow cytometry [16]. All protease inhibitors were used at non-toxic concentrations as assessed by exclusion of propidium iodide.

2.3. DNA analysis and cell size

Conventional and field inversion gel electrophoresis (FIGE) were carried out as previously described [5]. Cell sizing and electron microscopy were carried out as described [12,17].

3. Results

3.1. Thymocyte apoptosis induced by γ-irradation is inhibited by TLCK

Apoptosis can be induced in thymocytes by a range of agents including dexamethasone, etoposide, thapsigargin and staurosporine. Apoptosis caused by these stimuli can be inhibited by TLCK [12]. Here we report that, in addition, apoptosis induced by γ-irradiation in unsorted thymocytes was inhibited by TLCK (data not shown). Furthermore there was no difference in sensitivity to TLCK inhibition between predominantly proliferating (F1) and quiescent (F2) thymocytes (Fig. 1).

3.2. TLCK sensitive target is downstream of de novo protein synthesis

Transcription and translation are often required for thymocyte apoptosis [18], and TLCK is known to inhibit transcription. Therefore the possibility that TLCK was affecting these processes was examined by comparing its effects with those of cycloheximide. Apoptosis induced by γ-irradiation (Fig. 1) dexamethasone,

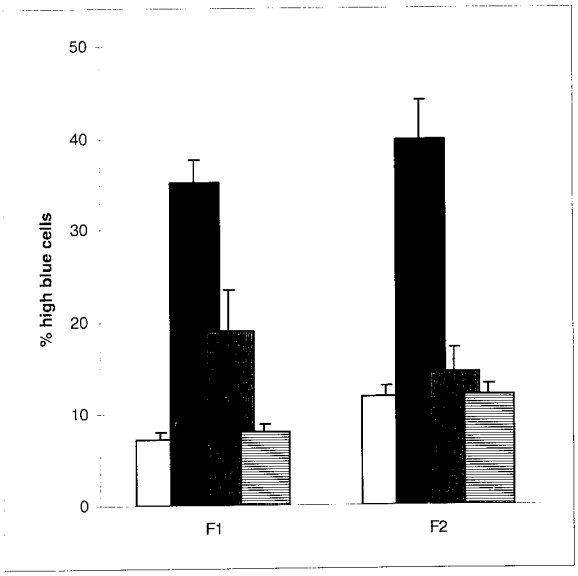


Fig. 1. Thymocyte apoptosis induced by γ-irradiation is inhibited by TLCK and cycloheximide. Primarily proliferating (F1) or quiescent (F2) thymocytes were incubated either alone (solid bars), with TLCK (50 μM; hatched bars) or with cycloheximide (10 μM; horizontal striped bars) for 1 h and subsequently irradiated (4 Gy) (Vickrad ^{60}Co source at 6.5 Gy/min). Cells were then incubated for a further 4 h and apoptosis assessed, as percentage high blue cells, by flow cytometry. The incidence of apoptosis in untreated cultures after 4 h incubation is also shown (open bars). The data are the mean (± S.E.M.) of 3 experiments.

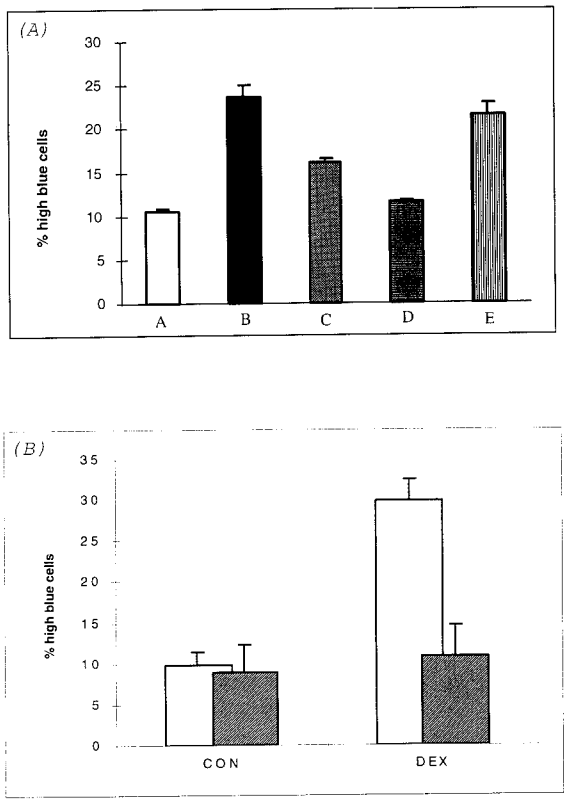


Fig. 2. (A) Staurosporine-induced thymocyte apoptosis is prevented by TLCK but not by cycloheximide. Thymocytes were incubated for 1 h either alone, with TLCK (50, 100 μM; C, D), or with cycloheximide (10 μM; E). Cells were subsequently treated for 4 h with staurosporine (1 μM; B). The incidence of apoptosis in untreated cultures is also shown (A). The results are the mean (± S.E.M.) of at least 3 experiments. (B) Z-VAD.FMK inhibits dexamethasone-induced apoptosis. Thymocytes were incubated for 1 h either alone (open bars) or with Z-VAD.FMK (200 μM; solid bars) and subsequently treated for 4 h with dexamethasone (DEX; 0.1 μM). The percentage of apoptotic cells (high blue cells) was assessed by flow cytometry. The data are the mean of at least 3 experiments (± S.E.M.).

etoposide and thapsigargin was inhibited by both TLCK and cycloheximide, whereas that induced by staurosporine was inhibited only by TLCK (Fig. 2A) [12]. These data together with our earlier studies [12] support a model in which the apoptotic machinery is pre-existing and staurosporine induces apoptosis by acting downstream of de novo protein synthesis but upstream of a TLCK sensitive target. Z-VAD.FMK inhibited dexamethasone-induced apoptosis (Fig. 2B). It also inhibited apoptosis induced by other stimuli (data not shown).

3.3. Lack of inhibition of DNA fragmentation by TLCK in isolated nuclei

In intact thymocytes TLCK inhibited both internucleosomal cleavage and the formation of large kilobase pair fragments of DNA induced by both dexamethasone and etoposide [12]. To ascertain whether proteolysis was involved before the initial cleavage of DNA that produces large fragments, the effects of TLCK on isolated rat thymocyte nuclei were investigated. TLCK binds and inactivates its target within 30 min [12]. To ensure a relevant TLCK concentration when testing the effects of TLCK on Mg^{2+}- and Ca^{2+}/Mg^{2+}-dependent chromatin degradation, cells were pre-treated with TLCK (50 μM), a

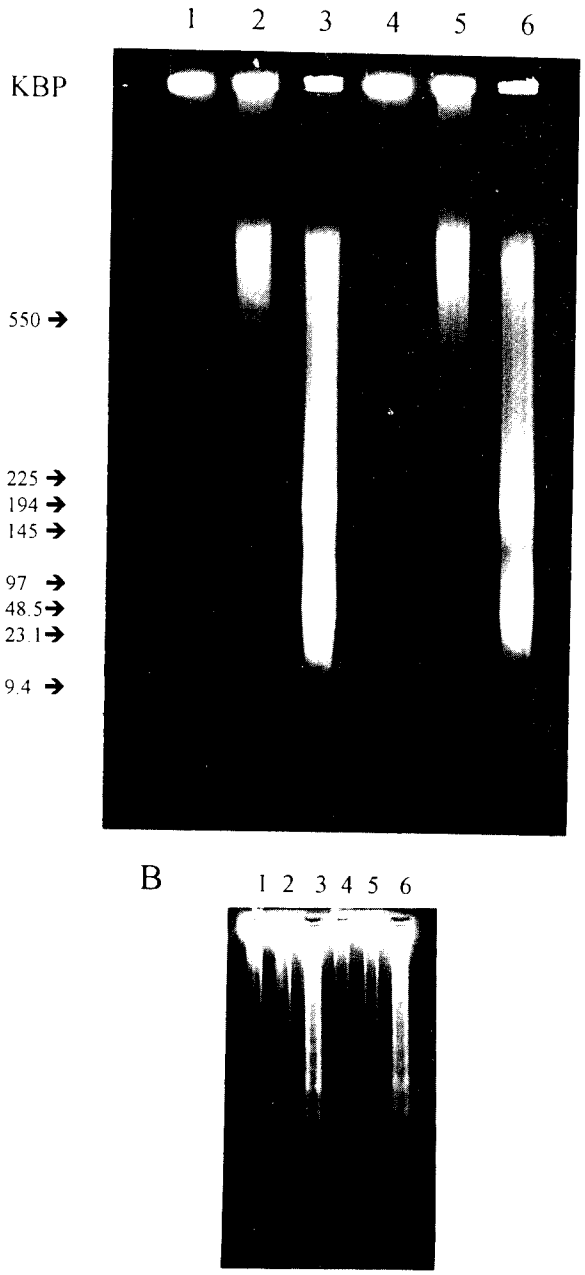


Fig. 3. The effect of TLCK on Mg^{2+}- and Ca^{2+}/Mg^{2+}-dependent DNA degradation in isolated nuclei. Thymocytes were incubated for 1 h either alone or with TLCK (50 μM). Nuclei from cells incubated alone were isolated and incubated in the absence of (lane 1) or presence of Mg^{2+} (lane 2) or Ca^{2+} and Mg^{2+} (lane 3). Nuclei from cells treated with TLCK were also incubated in the absence of (lane 4) or presence of Mg^{2+} (lane 5) or Ca^{2+} and Mg^{2+} (lane 6). DNA degradation was assessed by FIGE (A) or conventional agarose gel electrophoresis (B).

concentration which inhibited apoptosis in intact cells, prior to isolation of nuclei. DNA of isolated thymocyte nuclei was degraded in both a Mg^{2+}- and a Ca^{2+}/Mg^{2+}-dependent fashion (Fig. 3 lanes 2 and 3 respectively). Thymocyte nuclei isolated from cells treated with TLCK degraded their DNA in a similar manner (Fig. 3, lanes 5 and 6 respectively). These results suggest that in intact cells TLCK inhibits apoptosis at a stage prior to DNA fragmentation.

3.4. TPCK induces changes characteristic of early apoptosis

Ultrastructural examination of cells following 4 h incubation with TPCK (25 μM) revealed that most thymocytes (80%) displayed a distinct ultrastructure (Fig. 4), characterised by condensed chromatin abutting the nuclear membrane similar to that observed with cells treated with dexamethasone in the presence of zinc [17]. Several of the cells treated with TPCK showed disintegration of the nucleolus together with the formation of cytoplasmic vacuoles. These studies demonstrated that TPCK induced both biochemical and morphological changes associated

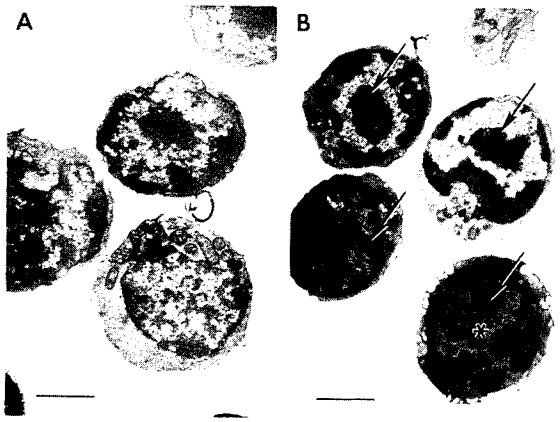


Fig. 4. TPCK causes perinuclear condensation of chromatin. (A) Thymocytes incubated in the control medium exhibited normal ultrastructure with diffuse aggregations of centrinuclear and perinuclear heterochromatin. (B) Cells incubated in the presence of TPCK (25 μM) showed condensation of heterochromatin into discrete, sharply-defined clumps (arrows). Several of these cells showed disintegration of the nucleolus (*) together with the formation of cytoplasmic vacuoles which fused with the cell membrane. Bars = 2 μm.

with early nuclear changes of apoptosis without producing internucleosomal cleavage.

4. Discussion

We and others have shown that there is an initial degradation of DNA to fragments of $\geqslant$700, 200–300 and 30–50 kbp in size [4,5,19]. These large fragments are precursors of DNA ladders and are associated with cells displaying an early apoptotic morphology [4,5,15,19]. Previously we have shown that TLCK inhibited the formation of these large fragments [12] consistent with proteolysis being required either for the initial cleavage of intact DNA to kilobase pair fragments or at some earlier stage in the apoptotic process. To distinguish between these possibilities, isolated thymocyte nuclei, which exhibit both a Mg^{2+}- and a Ca^{2+}/Mg^{2+}-dependent DNA degradation, were used as a model of the nuclear changes seen during apoptosis [20]. With these nuclei, TLCK inhibited neither the formation of large fragments nor internucleosomal cleavage (Fig. 3). These data were inconsistent with the first possibility and suggested that proteolysis was required at an early stage of apoptosis, prior to the formation of kilobase pair fragments of DNA.

4.1. TPCK inhibits DNA laddering but itself induces early apoptotic morphology

TPCK exerted markedly different effects compared with TLCK. TPCK inhibited internucleosomal cleavage induced by dexamethasone or etoposide [9,12] but alone caused the formation of large DNA fragments [12]. The induction of internucleosomal cleavage of DNA by dexamethasone or teniposide but not the formation of large fragments in rat thymocytes was prevented by TPCK [11]. On ultrastructural examination of TPCK-treated cells, formation of cytoplasmic vacuoles and condensation of chromatin into sharply defined clumps abutting the nuclear membrane were observed (Fig. 4). Both the pattern of DNA fragmentation and the morphology are typical of early nuclear changes of apoptosis [17,21]. Consistent with the alterations in cytoplasmic morphology, TPCK alone induced shrinkage in a proportion of cells [12]. These data demonstrate that the effects of TLCK and TPCK are different and that TPCK alone induces many of the early changes of apoptosis and only inhibits the terminal stages of DNA fragmentation, i.e. internucleosomal cleavage and full chromatin condensation.

4.2. Proteases and thymocyte apoptosis

In T-cell receptor-mediated apoptosis, in both a T-cell hybridoma and peripheral T cells, the effects of protease inhibitors have suggested the involvement of more than one protease and that these proteases are involved at different stages of the process [22]. The relationship between proteolysis and endonuclease activity was further examined by investigating the effects of protease inhibitors on the formation of large kilobase pair-sized fragments of DNA. In intact thymocytes some protease inhibitors prevented the conversion of large kilobase pair-sized DNA fragments into a DNA ladder but did not prevent the formation of fragments greater than 50 kbp in size [11]. The present study also showed that TLCK did not act directly on the calcium- and magnesium-dependent endonuclease(s).

The marked contrast between the effects of TLCK and TPCK on thymocyte apoptosis ([12] and this study) suggests roles for trypsin-like and chymotrypsin-like proteases at different stages of the apoptotic pathway. TLCK acts at a very early stage of the process, inhibiting all changes associated with apoptosis. TPCK prevents only the terminal biochemical and morphological changes induced by dexamethasone or etoposide. However, at the same concentration TPCK alone causes biochemical and morphological changes associated with early apoptosis (Fig. 4). TPCK clearly has 2 distinct effects and therefore is likely to have 2 distinct targets in the cell. One target is required for the progression of early apoptotic cells to a fully apoptotic phenotype. The data suggest that this target is nuclear, which is consistent with the results obtained from both thymocyte [11] and hepatocyte nuclei. Alkylation of a second target by TPCK produces many

characteristics of apoptosis. This effect of TPCK may be explained in 2 ways. A specific target may be a negative regulator of apoptosis and so its modification by TPCK releases the apoptotic machinery which had previously been held in check. Alternatively, alkylation by TPCK may be detected as non-specific damage by the affected cell which subsequently opts for controlled death rather than attempting repair.

4.3. A hierarchy of proteases

A requirement for different proteases has been implicated in T-cell receptor-mediated apoptosis [22]. Z-VAD.FMK inhibited apoptosis induced by dexamethasone (Fig. 2B) and by other stimuli (data not shown) suggesting the involvement of an ICE-like protease at an early stage of apoptosis. Thus the present results, together with our other studies ([12] and unpublished data), support the involvement of both an ICE-like and a trypsin-like protease at an early stage of thymocyte apoptosis and a chymotrypsin-like protease at a late stage of thymocyte apoptosis.

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Role of active cell death (apoptosis) in multi-stage carcinogenesis

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Abstract

Active cell death is a genetically encoded self-destruction of a cell. There occur morphologically different types of active cell death, e.g. apoptosis in the liver or autophagic cell death in human mammary carcinoma cells after tamoxifen treatment. (Pre)neoplastic lesions in rat liver exhibit enhanced rates of apoptosis, which tend to increase with increasing malignancy. Tumor promoters and non-genotoxic carcinogens inhibit active cell death, thereby increasing the accumulation of (pre)neoplastic cells and accelerating the development of cancer. On the other hand promoter withdrawal, fasting or application of negative growth signals such as transforming growth factor β1 (TGF β1) enhance apoptosis and can lead to selective regression of preneoplastic lesions or tumors.

Keywords: Apoptosis; Autophagic cell death; Non-genotoxic carcinogens; Tumor promotion; Liver; MCF-7 cells

1. Introduction

Active cell death is a genetically encoded active self-destruction of a cell. It serves as a counterpart of mitosis in the maintenance of an adequate cell number in tissues by elimination of cells which are no longer needed by the organism. It also serves to eliminate damaged cells. Active cell death can be induced by withdrawal of growth factors and trophic hormones and is controlled by the growth regulatory network of the organism [1–3]. Obviously, during carcinogenesis and tumor growth the regulation of cell proliferation and active cell death are disturbed. The present paper will review some recent experimental approaches and concepts to understand and define the underlying defects.

Active cell death can occur as morphologically different types. Apoptosis, currently the most widely known type, is characterized by condensation of cytoplasm and nucleus, abutting of chromatin masses (sometimes as crescents) at the nuclear membrane, fragmentation and heterophagy of residues by neighbouring cells. In contrast, during cell death type II formation of autophagic vacuoles and lysosomal activation are early and prominent events which are followed

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by pyknotic chromatin condensation. Further subtypes of active cell death have been described [4,5].

2. Autophagic cell death in mammary cancer cells

Estrogens have mitogenic and tumor-promoting activity in the mammary gland and in other tissues. Their effects are antagonized by anti-estrogens such as tamoxifen which is an important and effective drug in the therapy of human mammary cancer. It is not known to what extent the therapeutic effect occurs by induction of active cell death.

The human mammary cancer cell line MCF-7 provides a useful model to study the mechanism of anti-estrogen action. Previous studies revealed an inhibitory effect on DNA synthesis and enhanced cell death [5,6]. We have confirmed these results using tamoxifen, 4-hydroxytamoxifen and ICI 164384 [5,7]. Thus, anti-estrogens have anti-proliferative and anti-survival activity. We have also found that estrogen treatment up to 4 days after tamoxifen blocks the occurrence of cell death. This observation is important because it indicates the active nature of death (mitogen rescue) [7].

Detailed morphological studies by electron microscopy revealed that the preparation for cell death starts with formation of autophagic vacuoles which gradually degrades most of the cytoplasmic structures. At a later stage, nuclear changes become apparent which consist of formation of chromatin clumps distantly from the nuclear membrane, and finally pyknotic condensation in the center of the nucleus. Only a minority of nuclei in dying cells show changes familiar from apoptosis, namely chromatin condensation at the nuclear membrane and nuclear fragmentation. Thus, morphologically the type of cell death induced in MCF-7 cells by anti-estrogens clearly does *not* correspond to the definition of apoptosis, but rather to that of type II cell death or autophagic cell death [4,5,7].

In order to test for a specific role of autophagy in cell death of MCF-7 cells we have used 3-methyladenine, which is known to inhibit autophagy in liver cells [8]. As shown in Table 1, treatment of cells with 3-methyladenine largely prevented the increase of cell death occurring 4 and 5 days after addition of tamoxifen to the culture. Remarkably, both types of nuclear alteration, namely apoptosis-like and pyknotic changes, were prevented. This finding supports the morphological data suggesting that enhanced autophagy is an early step on the pathway to type II cell death.

3. Apoptosis in stages of liver cancer

Carcinogenesis in rat liver, as in other organs, occurs as a stepwise process via sequential intermediate cellular phenotypes from normal to malignant cells [9,10]. The first step or initiation can be induced by genotoxic insults and may result from mutational events; it may also occur for unknown reasons ('spontaneously') as in most human cancers. Initiation usually seems to occur in single cells which have a proliferation

Table 1
Inhibition by 3-methyladenine of active cell death in human mammary carcinoma cells MCF-7

Day after 3-MA	Tamoxifen		Tamoxifen + 3-MA	
	Pyknotic nuclei	Crescent and fragmented nuclei	Pyknotic nuclei	Crescent and fragmented nuclei
0	2.83 ± 0.29	1.72 ± 0.83		
1	3.9 ± 0.07	3.85 ± 0.35	1.45 ± 0.21	1.15 ± 0.21
2	6.4 ± 0.82	3.37 ± 0.61	2.7 ± 0.42	2.7 ± 1.41

0 = day of 3-methyladenine (3-MA) treatment, 10 mM. 3-MA was added 3 or 4 days after tamoxifen (10^{-6} M). Means (±SD) of 3 representative experiments are given. Dead cells were identified by nuclear morphology after staining with Hoechst 33258 and counted by light microscopy. Because of their low incidence, nuclei with crescent chromatin and fragmented nuclei were added together.

advantage over normal cells and thereby can grow into larger clones. The growth rate of initiated cells and cell clones, and thereby cancer development, is accelerated by tumor promotion. Further mutations in these clones may give rise to new clones with higher growth advantage that eventually may form malignant tumors (progression). Rodent liver currently offers one of the best model systems to study the stepwise development of cancer because putative initiated, preneoplastic and adenomatous cells can be identified by a variety of markers, and their biological properties have been well characterized [9,10].

The growth rate of cell populations depends on rates of cell replication (α) and rates of cell death (β). The resultant ($\alpha - \beta$) determines the growth rate of the cell population. It is sometimes assumed that tumor growth ensues from a decrease in death rates of cells. However, this concept does not apply to the liver and a number of other organs (see below).

When the death rate of cells is >0 ($\beta > 0$) there is a probability of extinction of the clone which is expressed as β:α [11]. This probability also depends on the size of the clone. Therefore it was predicted by Luebeck et al. that the majority of initiated cells and small preneoplastic clones may be extinguished [11]. In fact, we and others have shown that as many as 80% of single putative initiated cells forming in rat liver after a single dose of a genotoxic carcinogen disappear again [2,3]. Therefore the effect of initiation can be reversed by active cell death.

Earlier studies of our group led to the discovery that putative preneoplastic cell foci in rat liver not only exhibit 5–10-fold higher rates of cell replication than normal hepatocytes but also enhanced rates of cell death by apoptosis. As a result, foci show little if any growth during early stages of carcinogenesis [12,13]. Tumor promoters were found to act as survival factors for preneoplastic cells. Thereby they accelerate the accumulation of those cells and favor development of cancer [2,3,12,13]. Even higher rates of cell replication and of apoptosis are found in hepatic adenoma and in liver cancer [3]. Likewise, human hepatocarcinoma on average exhibit manyfold higher rates of both cell birth and cell death than the surrounding tissue [3]. Obviously, the enhanced incidence of apoptosis in preneoplastic cells is not eliminated by selection in the course of tumor progression. Increased birth and death rates of cells will enhance the chance to acquire additional genetic alterations and may thus provide a selection advantage in the cell population. The molecular basis of why preneoplastic and neoplastic cells exhibit higher rates of replication and death is not known but it is of interest that some immediate-early genes such as *fos*, *myc*, *jun*, etc. may be involved in intracellular signal cascades that lead to cell replication *and* to death [2,3,14,15].

Functional implications of these findings include that a (pre)neoplastic cell population will show selective growth upon stimulation by mitogenic agents or survival factors. It is most intriguing, however, that the same population should show selective *regression* with growth-inhibitory and death-inducing signals (see illustration in Fig. 1). We have recently shown that food restriction in rats for 3 months to 60% of normal results in an almost selective loss of preneoplastic cell foci from the liver. Foci decreased by 85% while normal liver cells decreased by only 20%. Obviously, preneoplastic cells are much more susceptible to negative growth signals exerted by food restriction than normal hepatocytes [16]. These findings also

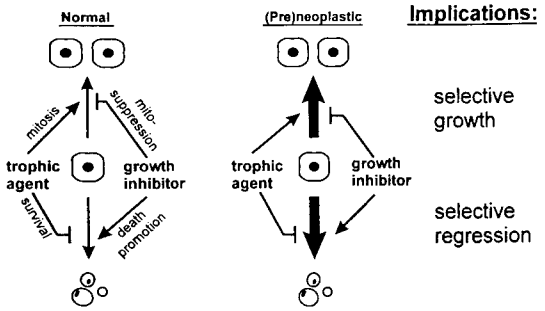


Fig. 1. Homeostasis of cell number in tissues by mitosis and active cell death and the effects of trophic agents and of growth inhibitors. Also shown is the overresponse of preneoplastic and tumor cells to trophic agents and growth inhibitors which may lead to selective growth or selective regression of tumors.

provide a new explanation for the well-known protection from cancer exerted by low calorie diets in both animals and humans.

Also, manifest liver tumors may show selective regression in response to negative growth signals. We have produced tumors by long-term treatment with the non-genotoxic liver carcinogen and tumor promoter nafenopin in rat liver. Upon withdrawal of nafenopin hepatic foci, adenoma and carcinoma showed extensive regression, by cessation of cell proliferation and enhanced active cell death [3].

4. Signal factors for active cell death in the liver

Signal factors involved in the induction of active cell death in the liver as well as in other organs include transforming growth factor β1 (TGF β1), activin, the fas-ligand and tumor necrosis factor [2,3,17]. We have demonstrated previously the role of TGF β1 in hepatocyte apoptosis. TGF β1 appears to be synthesized in hepatocytes preparing for apoptosis [18], and injection of TGF β1 into intact animals induced enhanced apoptosis. The effect of TGF β1 injection was synergistically enhanced if it was preceded by a period of hepatomitogen treatment followed by mitogen withdrawal [19]. Thus, a state of 'mitogen deficiency' may predispose cells for induction of apoptosis by TGF β1. In an attempt to block endogenous estrogens which act as mitogens in the liver, we have treated rats with tamoxifen, and the effect on apoptosis, with and without TGF β1, in normal liver and in preneoplastic foci was investigated. The results presented in Fig. 2 show that indeed tamoxifen and TGF β1 synergistically enhanced the incidence of apoptosis. Furthermore, cells in foci were much more susceptible to this effect than normal liver cells [20]. As a result, foci showed selective (preferential) regression as compared to the normal liver (Fig. 2, see above).

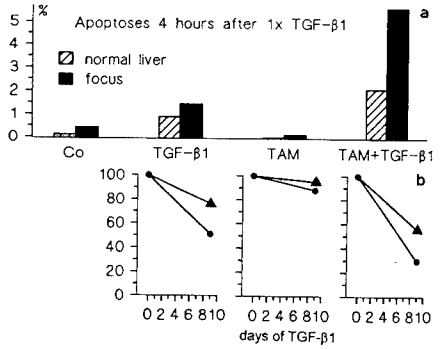


Fig. 2. Effects of TGF β1 and tamoxifen (TAM) on apoptosis and volume of preneoplastic foci in the liver. Female rats received a single dose of 130 mg/kg dimethylbenzanthrazene. From 13 weeks later tamoxifen (8 mg/kg daily) was given s.c. TGF β1 (40 μg/kg) was injected i.v. either once (a) or once daily for 8 days (b). Putative preneoplastic foci were identified with antibodies to glutathione *S*-transferase P, and the incidence of apoptosis was determined as described previously (14). Ordinates in (a): incidence of apoptoses; in (b): % change of liver DNA (▲) and foci volume (●). Values at day 0 (●) were set 100%. DNA was assayed as an index of cell no. in the total liver. Note that foci volume declines more than total liver DNA indicating preferential regression of foci.

5. Implications

Active cell death is involved in all major stages of cancer development. Some implications of these findings are compiled in Table 2. For understanding mechanisms of action of carcinogenic agents it is important to recognize that non-genotoxic carcinogens may have 2 different effects on their target cells: they may either increase replicative activity, or they may act as survival factors and prevent cell elimination. Due to the increasing susceptibility to death signals of (pre)neoplastic altered cells, non-genotoxic carcinogens may induce the more rapid accumulation of cells, the more advanced the lesions are.

A further conclusion relates to the mode of feeding in long-term testing of compounds for carcinogenicity. Our data suggest that feeding experimental animals ad libitum stimulates growth rates of preneoplastic lesions, in the liver and possibly in other organs. This may exaggerate the sensitivity of animals to weak carcinogenic stimuli and may even lead to false-positive results. On the other hand if the compound under study reduces food consumption some protection from cancer development may

Table 2
Active cell death in tumor development. Implications for understanding mechanisms of action of non-genotoxic carcinogens and for risk assessment of chemicals

Initiation:	(a) Extinction of initiated cells by active cell death → Reversibility of initiation possible (b) Promoters act as survival factors for initiated cells → 'Co-initiation'
Promotion:	(a) Promoters act as survival factors for (pre)neoplastic cells → Accumulation of initiated/preneoplastic cells (b) Promoter withdrawal → Reversibility of promotion (c) Growth inhibitors will lead to anti-promotion
Progression:	Enhanced cell turnover → increased probability to accumulate genetic alterations
Tumor growth:	(a) Limited by active cell death (b) Depends on continued availability of non-genotoxic carcinogen/promoter/survival factor (c) Withdrawal of non-genotoxic carcinogen or treatment with anti-hormones/anti-mitogens may cause 'selective regression'

result and may weaken or even mask a possible carcinogenic potential of that compound.

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Toxicology Letters

Signalling mechanisms and oxidative stress in apoptosis

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Abstract

A variety of stimuli can induce cells to undergo apoptotic death. One of the most reproducible inducers is mild oxidative stress, be it via exposure to hydrogen peroxide, redox-cycling quinones or thiol-alkylating agents. Oxidative modifications of proteins and lipids have also been observed in cells undergoing apoptosis in response to non-oxidative stimuli such as glucocorticoids or topoisomerase II inhibitors. This suggests that some unidentified oxidative changes occur during apoptosis in many, if not all, cases. However, recent experiments demonstrating apparently normal apoptosis even when cells are cultured at low oxygen tensions show that reactive oxygen species cannot be essential mediators of this type of cell death. Experiments revealing that apoptosis is typically accompanied by a depletion of intracellular reduced glutathione (GSH) are also discussed. As GSH depletion will lower a cell's capacity to buffer against endogenous oxidants, we propose that it contributes to the increased oxidative damage commonly observed to accompany apoptosis. In addition, it may set a time limit on continued mitochondrial function (and thus indirectly on total ATP levels and membrane integrity) in apoptotic cells, and thereby explain the often observed 'secondary necrosis' of cells undergoing apoptosis in vitro.

Keywords: Thymocyte; ROS; Glutathione; ICE-like proteases; Dithiocarbamate; Necrosis

1. Introduction

Apoptosis is a form of cell death in which an individual cell undergoes an internally controlled transition from an intact metabolically active state into a number of shrunken remnants retaining their membrane bound integrity [1,2]. Lysis of internal organelles apparently does not occur during this process, and little external leakage of the contents of the dying cell can be detected. As a consequence, apoptotic cells do not induce an inflammatory response in vivo. Instead the shrunken apoptotic bodies are phagocytosed and their contents presumably recycled [3]. Apoptosis therefore provides an organism both with a safe and efficient way to continuously turnover cells in any tissue, and the capability to remove specific cells during development.

Genetic and biochemical investigations of apoptosis in cells from various organisms have identified several highly conserved protein families specifically involved in the molecular mechanism of this form of cell death. A gene product called *bcl-2* first identified in human B-cell lymphomas as a common translocation from its normal site on chromosome 18 to a region close to the immunoglobulin heavy chain promoter on chromosome 14 is homologous to a

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negative regulator of cell death (*ced-9*) found in the nematode *Caenorhabditis elegans*. Overexpression of *bcl-2*, or the related gene *bcl-x*$_L$, protects a variety of mammalian cells from undergoing apoptosis induced by many different stimuli [4]. Another *C. elegans* gene product called *ced-3* was identified as being essential for the occurrence of developmental cell death. Again the protein encoded for by this gene has significant homology to a family of mammalian proteins, in this case cysteine proteases related to interleukin 1-β converting enzyme (ICE). Overexpression of several of these proteases can induce apoptosis in mammalian cells, while inhibition of their activity either with non-hydrolysable peptide analogs or co-expression of the viral ICE-protease inhibitor crm-A potently inhibits apoptotic cell death [5]. It thus appears that protease activation and subsequent cleavage of as yet unidentified protein substrates is a common event in many, if not all, forms of apoptosis.

Oxidants including reactive oxygen species (ROS), lipid hydroperoxides and nitric oxide are also believed to be widely involved in apoptosis. Much of the evidence for this comes from numerous observations that antioxidants inhibit/delay the onset of this type of cell death. For example, cultured sympathetic neurons die by apoptosis when deprived of nerve growth factor (NGF). Direct injection of neurons with either copper/zinc superoxide dismutase (SOD) protein or cDNA within the first few hours of NGF removal delays cell death, while significant increases in the intracellular peroxide tonus can be detected within 3 h [6]. Similar results have been obtained with an interleukin 3-dependent pro-B lymphocyte cell line. In these cells, growth factor withdrawal induces apoptosis while overexpression of glutathione peroxidase (but not SOD) provides significant protection [7]. In each example, successful transmission of a physiological signal for cell death is apparently partly dependent on subsequent oxidative changes within the receiving cell. However, this does not imply that ROS alone are absolutely required as mediators of all types of apoptosis. Three independent publications have recently shown that various forms of apoptosis can occur with approximately similar efficacy independent of ambient oxygen tension [8–10], while the interaction between FAS ligand and its receptor is one physiologically important stimulus for apoptosis that is not influenced by the presence of various antioxidants and metal chelators [11,12]. In this review we attempt to dissect the controversy surrounding 'oxidative stress and apoptosis', and emphasise how some of the redox changes observed in dying cells may relate to the overall apoptotic process.

2. Pro-oxidant conditions promote either apoptosis or necrosis

Direct exposure of cells to oxidants such as hydrogen peroxide or redox-cycling quinones causes multiple intracellular alterations, including elevation of cytosolic Ca^{2+}, depletion of ATP, and oxidation of NADH, reduced glutathione (GSH), and lipids [13]. Necrosis is the characteristic end point of such a dramatic disturbance in cell homeostasis, and as apoptosis and necrosis represent such distinct pathways to cell death the possibility that lower concentrations of oxidants could also promote apoptosis was initially ignored. One of the first studies reversing this neglect was undertaken by Lennon et al., who observed that low amounts of hydrogen peroxide induce apoptosis in HL-60 cells, while necrosis occurs when millimolar concentrations of the oxidant are present [14]. Exposure of RINm5F cells to increasing doses of redox-cycling 2,3-dimethoxy-1,4-naphthoquinone progressively results in cell proliferation, apoptosis and finally necrosis [15]. Oxidised low density lipoproteins and lipid hydroperoxides such as 15-hydroperoxyeicosatetraenoic acid are other oxidants reported to induce apoptosis when directly applied to cells [16,17]. Finally, mild oxidation of intracellular sulphydryls with low concentrations of diamide induces apoptosis in T cells [18]; raising the concentration of the oxidant was again observed to shift the form of cell death away from apoptosis towards necrosis.

Dithiocarbamates are thiol-containing molecules with a diversity of applications, both experimentally (they are often used as antioxidants

in cell biological studies) and commercially (as an important class of agricultural pesticide with additional minor clinical utilities). We have recently studied the effects of these compounds, in particular pyrrolidine dithiocarbamate (PDTC), on rat thymocytes (Nobel, Kimland, Lind, Orrenius and Slater, J. Biol. Chem. in press). Exposing these cells to PDTC (1–40 μM) induces an initial 2–3-fold increase in the intracellular concentration of glutathione disulfide (GSSG), which is followed several hours later by the cell shrinkage and chromatin fragmentation that typifies apoptosis in thymocytes. Inclusion of membrane-impermeable metal chelators in the incubation medium abrogates these effects, while analysis of intracellular metals revealed a rapid 8-fold elevation of copper in the treated cells. In contrast, intracellular iron levels were unchanged. Supplementation of the culture medium with traces of copper was also found to potentiate toxicity. As dithiocarbamates are well known to be lipophilic compounds possessing strong copper-chelating properties, these results suggest that intracellular transport of redox-active copper promoting an oxidative stress within the treated cells causes the subsequent onset of apoptosis. Our studies with PDTC thus provide a particularly clear example of an environmental toxicant inducing apoptotic cell death via an oxidative shift in intracellular redox state.

3. Evidence for oxidative changes during thymocyte apoptosis

While it is now widely accepted that oxidative stress is one of many stimuli capable of promoting apoptosis, it is much less clear whether any common oxidative changes universally accompany apoptotic cell death. The experiments described above demonstrating apparently normal apoptosis in a low-oxygen environment suggest that normal/enhanced production of ROS is not an absolute requirement for this type of cell death. However, neither oxidation nor changes in intracellular redox state (for example of protein sulphydryls or GSH) are synonymous with molecular oxygen and its metabolites. It should also be noted that approximately equivalent oxidative modification to DNA and bulk protein was detected between cells undergoing apoptosis in normoxic and hypoxic conditions (Fig. 3 in [10]). Apoptosis is a complex cellular event involving at the very least the coordinated action of several different proteases, nucleases and membrane-associated ion channels and phospholipid translocases, and it is reasonable to speculate that some of these events will be subject to redox control.

Thymocytes are a convenient model system for studying apoptosis. In vivo these cells rapidly undergo negative selection by apoptosis, and they are also sensitive to experimental induction of apoptosis in a variety of ways. Both we and others have observed that antioxidants as diverse as metal chelators, nitrone free-radical spin traps and the endogenous thiol reductant dihydrolipoic acid (DHLA) each inhibit thymocyte apoptosis induced by stimuli as different as glucocorticoid hormones, DNA-damaging agents or Ca^{2+} ionophores [19,20]. With respect to DHLA, this inhibition was evident before any shrinkage of the cells could be detected, while chromatin fragmentation was blocked beyond the initial 300 kbp stage [20]. Density gradient centrifugation can be used to isolate sub-populations of thymocytes at different stages of apoptosis [21], and this method was therefore employed to investigate possible oxidative changes occurring in these cells. Both pre-apoptotic (partially shrunken cells which contain high-molecular-weight chromatin fragments) and fully apoptotic cells have a lower GSH, protein sulphydryl and α-tocopherol content than thymocytes of normal density [20]. Similar changes are seen irrespective of whether apoptosis is induced by a glucocorticoid hormone or occurs spontaneously in culture, and they thus seem to be consistent phenomena accompanying apoptosis in these cells. However, even though nitrone free-radical spin traps prevent these changes and inhibit cell death, we have been consistently unable to detect any electron spin resonance signal from ROS in thymocytes treated with apoptosis-inducing agents. This may reflect the inherent insensitivity of the method, although the experiments at low oxygen tension described above suggest an alternative explanation: ROS are not crucial,

even though some other event resulting in a depletion of intracellular GSH and reduced protein thiols does occur.

4. GSH depletion also occurs during apoptosis in many other cell systems

Intracellular GSH levels can be artificially lowered in several ways, for example by inhibiting its biosynthesis with buthionine sulfoximine, binding free sulphydryl groups with diethyl maleate or simply withdrawing cystine from the cell culture medium. When treated in this way, cells can usually tolerate GSH levels down to about 20% of normal, although they are usually very sensitive to any additional stress. Apoptosis can still be induced in GSH-depleted cells, although necrotic lysis tends to predominate [22,23]. Lowering GSH levels alone is therefore clearly insufficient to induce apoptosis.

However when GSH levels are measured as thymocytes undergo apoptosis in response to glucocorticoids or etoposide, the concentration of the thiol is seen to fall approximately coincident with chromatin degradation and cell shrinkage [20]. As mentioned above, density-gradient centrifugation reveals that these changes are confined to both pre-apoptotic and apoptotic cells. Similar changes were seen both when murine L929 fibroblasts were treated with tumour necrosis factor-α and when anti-FAS antibody was added to human JURKAT T cells (unpublished data). Experiments are in progress to determine the fate of the missing GSH; is it oxidised into GSSG and then exported from the cell, degraded or covalently reacted with proteins as a mixed disulphide?

Jacobson et al. have presented a model for apoptosis in which an unidentified cytoplasmic control system (a cascade of ICE-like proteases?) orchestrates the multiple downstream events (such as cell shrinkage and chromatin degradation) that comprise apoptosis [24]. We postulate that a loss of cytoplasmic (and nuclear?) GSH is one of these characteristic downstream changes. A loss of cytoplasmic GSH will influence the redox buffering capacity of the cell, such that it becomes less able to tolerate the presence of oxidants. When mitochondrial GSH is also eventually depleted (this pool is significantly more resistant to depletion compared with the cytosol) energy production will be affected. The cell would consequently swell and undergo a 'secondary necrosis'. Such an outcome is typically observed in cell culture studies performed in the absence of phagocytes. We thus propose that one function of a GSH depletion during apoptosis is to set a limit on how long the apoptotic cell can persist in the absence of phagocytosis. Development of this hypothesis will require us to determine whether a GSH depletion also occurs when cells undergo apoptosis at low oxygen tension; if so, then it will imply that this is an oxidative change that occurs independent of a cell's capacity to produce ROS.

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Bcl-2 family proteins: regulators of chemoresistance in cancer

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Abstract

Knowledge about the mechanisms that regulate the expression of the family of *BCL*-2 genes and the biochemical characteristics of their encoded proteins is beginning to provide new insights into the origins of cancer and our all too often inability to adequately treat it. With better understanding of the functional significance of these protein-protein interactions involving Bcl-2, Bax, and other members of the Bcl-2 protein family and with insights into the structural details of these interactions, it may eventually be possible to develop novel pharmacological agents that improve tumor responses to currently available anticancer drugs and that have other clinically relevant uses as well.

Keywords: Bcl-2 protein; p53; Cancer; Apoptosis

1. Introduction

The *BCL*-2 gene was first discovered because of its involvement in the t(14;18) chromosomal translocations found in most follicular non-Hodgkin's B-cell lymphomas, a low-grade neoplasm characterized by a gradual accumulation of G_0/G_1-phase mature B cells which arises in ~20 000 new patients per year and has a prevalence of ~150 000 cases in the United States population alone (reviewed in [1]). *BCL*-2 contributes to neoplastic cell expansion by blocking apoptosis caused by the physiological cell death mechanisms that normally help to maintain cell numbers at homeostatic levels in essentially all tissues with self-renewal capacity. Since its initial discovery in lymphomas, evidence has been obtained which suggests that *BCL*-2 gene expression becomes dysregulated in a wide variety of human cancers, probably through mechanisms that involve alterations in the transacting factors that control the expression of this anti-apoptotic gene rather than because of gross structural alterations to the gene such as the chromosomal translocations seen in lymphomas (reviewed in [2]). In addition to *BCL*-2, several homologous genes have been discovered, some of which block cell death and others of which promote apoptosis [1,2]. Knowledge about the mechanisms that regulate the expression of these genes and the biochemical characteristics of their encoded proteins is beginning to provide new insights into the origins of cancer and our all too often inability to adequately treat it.

2. *BCL*-2 is a determinant of chemoresistance

Essentially all currently available anticancer drugs, as well as γ-radiation, ultimately kill

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cancer cells by triggering apoptosis, probably through activation at least in part of the same biochemical pathways normally utilized in vivo for control of cell numbers by programmed cell death. Gene transfer experiments have demonstrated that elevated levels of Bcl-2 protein can protect tumor cells from induction of apoptosis by a wide variety of chemotherapeutic drugs and radiation [3,4]. Conversely, antisense-mediated reductions of Bcl-2 protein levels can render tumor cells more sensitive to induction of apoptosis by cytotoxic anticancer drugs [5,6]. These observations, as well as the results of several clinical-correlative studies where Bcl-2 protein levels in tumor specimens were correlated with patient responses to therapy, have suggested that the relative levels of Bcl-2 can be an important determinant of the relative sensitivity or resistance of cancer cells to currently available therapeutics.

More recently, we have begun to analyze the expression of other members of the *BCL-2* gene family in human cancers. These studies suggest that alterations in the expression of other members of this multigene family can occur and may be associated with a chemoresistant phenotype in some circumstances. For example, marked reductions in the expression of the pro-apoptotic gene *BAX* were detected by immunohistochemical methods in approximately one-third of adenocarcinomas of the breast. In a cohort of ~120 patients with metastatic disease, reduced *BAX* expression correlated with poor responses to combination chemotherapy, faster time to tumor progression, and shorter overall survival [7]. Conversely, striking upregulation of the levels of the anti-apoptotic protein Bcl-X_L have been detected in drug-resistant acute myeloid leukemia (AML) cell lines [8,9].

3. Mechanisms of regulating *BCL-2* family gene expression: the p53 connection

Overall, relatively little is known about the molecular mechanisms that control the expression of *BCL-2*, *BAX*, and other members of the *BCL-2* gene family. Recently, however, we demonstrated that the p53 tumor suppressor gene product can function as a transcriptional repressor of *BCL-2* and as a transcriptional activator of the *BAX* gene [10–12]. For example, in a murine myeloblastic leukemia line M1 which lacks p53, conditional restoration of p53 using a temperature-sensitive mutant resulted in a rapid reduction in *BCL-2* mRNA levels and a simultaneous elevation in *BAX* mRNA [10]. Temperature-dependent restoration of p53 activity in these cells also resulted in apoptosis. Using reporter gene assays and a human lung cancer line H358, we also mapped a p53-dependent negative response element (NRE) in the human *BCL-2* gene to an ~200-bp segment located in the 5′-untranslated region (5′-UTR) of the gene [11]. This p53-dependent NRE functions in both a position- and orientation-independent fashion to suppress the activity of heterologous promoters in a p53-dependent manner, suggesting that it has properties consistent with a transcriptional silencer element. For studies of *BAX*, we cloned the human *BAX* gene. Sequencing of the *BAX* promoter region revealed the presence of 4 sites with strong homology to the decameric p53-binding site consensus sequence, clustered into a region of 39 bp and located a few hundred base pairs upstream of the TATAA box and principal transcription initiation site. In reporter gene assays using *BAX*-CAT constructs and a variety of p53-deficient tumor lines, p53 strongly transactivated transcription via an ~300-bp *BAX* promoter fragment that contained the putative p53 binding sites [12]. In vitro, recombinant p53 protein bound to oligonucleotides comprising the 39-bp sequence from the *BAX* gene that contained the 4 decameric sequences with homology to consensus p53 binding sites.

To explore the in vivo relevance of p53 for regulation of *BCL-2* and *BAX* gene expression, we analyzed the relative levels of the Bcl-2 and Bax proteins in a wide variety of tissues using immunoblotting and immunohistochemical assays [10]. Those studies revealed marked elevations in Bcl-2 and reductions in Bax protein in several tissues, but not in most organs examined. Thus, while p53 represents one of the potential inputs into the *BCL-2* and *BAX* genes, it clearly

is not the only transacting factor that influences the expression of these genes and, hence, the relative role of p53 in controlling Bcl-2 and Bax protein levels is highly tissue-specific. In this regard, more recent reporter gene analysis of the additional segments of the *BAX* promoter in our laboratory has revealed the presence of negative regulatory elements that substantially impair the ability of p53 to transactivate the *BAX* promoter (unpublished data). Furthermore, we have observed in γ-irradiated mice that rapid elevations in Bax protein levels occur in lymphoid tissues and small intestinal crypt cells, but not in most other tissues [13]. Since γ-radiation is known to induce p53-dependent elevations in p21-WAF-1 in nearly all tissues [14], whereas γ-radiation induces *BAX* only in a subset of tissues, these results imply that p53 is capable of transactivating *BAX* only in some tissues. Interestingly, the cell types in which radiation induces increases in Bax protein levels in vivo are also exquisitely radiosensitive and undergo apoptosis in response to radiation. In contrast, the tissues in which Bax increases are not induced fail to experience cell death in γ-irradiated animals and are generally regarded as radioresistant. Some of these observations on Bax regulation by γ-irradiation have been translated to human tumor lines in vitro, revealing a strong correlation between ability of γ-irradiation to induce Bax and stimulate apoptosis. Tumor lines in which radiation did not induce *BAX* did not undergo apoptosis but did experience cell cycle arrest and were induced to express 21-WAF-1, provided that p53 was wild-type [15]. Taken together, these observations suggest that the ability of p53 to induce increases in the expression of *BAX* profoundly influences the response of tumor cells to radiation, and determines ultimately whether the outcome will be cytotoxicity versus merely cytostasis.

4. Protein-protein interactions involving the Bcl-2 family

Many members of the Bcl-2 protein family are capable of directly interacting with each other through a network of homo- and heterodimers. The Bcl-2 protein, for example, can homodimerize with itself, and heterodimerizes with Bax, Bcl-X_L, Bcl-X_S and Mcl-1 [16,17]. The functional significance of most of these protein-protein interactions remains enigmatic to date. The ability of Bcl-2 to heterodimerize with Bax, however, has been correlated with function as a suppressor of apoptosis, in as much as mutants of Bcl-2 that fail to bind to Bax but which are still capable of binding to wild-type Bcl-2 protein are essentially inactive where blocking of apoptosis is concerned [18]. On the other hand, some mutants of Bcl-2 that still retain the ability to bind to Bax are nevertheless biologically inactive, suggesting that perhaps Bcl-2 must perform other functions besides heterodimerizing with Bax in order to suppress cell death [19]. In this regard, we have recently shown that the NH_2-terminal first 82 amino acids of Bcl-2 are not required for binding to Bax but do appear to be necessary for interactions (either directly or indirectly) with other Bcl-2 binding proteins such as BAG-1 and Raf-1, both of which have been shown to synergistically cooperate with Bcl-2 in suppressing apoptosis [20,21]. With better understanding of the functional significance of these protein-protein interactions involving Bcl-2, Bax, and other members of the Bcl-2 protein family and with insights into the structural details of these interactions, it may eventually be possible to develop novel pharmacological agents that improve tumor responses to currently available anticancer drugs and that have other clinically relevant uses as well.

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Modulators of signal transduction as cancer chemotherapeutic agents – novel mechanisms and toxicities

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Abstract

The recent explosion of knowledge of the molecular basis of regulation of growth and differentiation has engendered the search for novel anticancer drugs which act via interference with cellular signal transduction pathways. Among the agents which are under investigation are modulators of protein kinases, for example quercetin and bryostatin 1. Both compounds have undergone phase 1 clinical evaluation. The major dose-limiting toxicities of quercetin and bryostatin are nephrotoxicity and myalgia, respectively. Novel analogs of the microbial product staurosporine, a potent kinase inhibitor, possess intriguing specificities for different kinases.

Keywords: Anticancer drugs; Kinases; Drug toxicity

Conventional chemotherapy of the major solid cancers is of limited success. The toxic side effects of such treatments are often life-threatening. Conventional cytotoxic drugs usually interfere with DNA, RNA or synthesis of structural proteins and affect normally proliferating cells such as those of the gut and bone marrow. The death of these cells is a side effect typical of cytotoxic therapy. Therefore potential antiproliferative treatments which would avoid toxicities of this type are of considerable interest. Among new targets currently explored for cancer teatment are oncogenes, tumor suppressor genes, anti-apoptotic and mitogenic signals. Many cellular signal transduction pathways are altered in malignant tissues. Therefore agents which affect signalling constitute an area of intense research in experimental cancer chemotherapy [1]. Signalling pathways from cell surface receptors to the nucleus, which mediate growth stimuli, involve chains of intercommunicating proteins, each of which integrates signals from upstream activators and passes them on to various downstream targets or effector proteins.

It is safe to predict that in a few years time cell-permeable peptides and therapeutic genes will enter the oncologists' therapeutic armamentarium, but undoubtedly new small molecules will also play a significant role, particularly as modulators of gene transcription. It is also safe to assume that these novel therapies will pose novel toxicological challenges. It is the aim of this presentation to focus on molecules of potential clinical interest in cancer therapy which modulate cell signalling. The legitimacy of targeting the cellular signalling machinery with the aim of curing a specific disease has been highlighted by the immunosuppressant agents cyclosporine and FK506 [2]. They act as molecular glue forming a complex with endogenous

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cyclophylins. This complex inhibits the phosphatase action of calcineurin, an important effector in T-cell activation, via the transcription factor NF-AT. Whilst the discovery of cyclosporine and FK506 and their mechanism of action was fortuitous, it nevertheless illustrates that it is possible to affect a specific disease by targeting cell signalling.

1. Agents targeting the Ras pathway

The signalling pathway which leads via the protooncogene product Ras (Fig. 1) is a plausible target for new anticancer drugs. This pathway is central to growth control in most organisms. Ras proteins bind guanine nucleotides and function like a binary 'switch'. In the 'off' position Ras binds guanine dinucleotide phosphate (GDP) and sits at the inner surface of the cell membrane. On activation it sheds GDP, binds guanine trinucleotide phosphate (GTP) and exists for a short time in the 'on' state, releasing a pulse of growth-stimulating signals into the cell. The bound GTP is rapidly converted to GDP by activation of the Ras protein's intrinsic GTPase activity. The Ras signal transduction cascade starts with the binding of a growth factor, such as epidermal growth factor (EGF) to its tyrosine kinase receptor, resulting in autophosphorylation of the receptor tyrosine residues. The principle target downstream of Ras is the Raf protein, which is also a kinase and the product of another oncogene. Once activated Raf phosphorylates a second kinase, which in turn controls mitogen-activated (MAP) kinase. Eventually the cascade leads to the phosphorylation of transcription factors which interact with DNA and regulate the expression of genes pivotal for proliferation and differentiation. Mutations which render Ras constitutively active have been found in a wide variety of human tumors. A post-translational modification which is necessary for the functioning of Ras is the addition of a farnesyl moiety to the cysteine residue of the C-terminal tetrapeptide sequence. Inhibitors of the enzyme protein farnesyltransferase are under consideration as potential inhibitors of *ras*-dependent transformation, which may be useful in the treatment of those cancers in which Ras plays a role. The tetrapetide 2(*S*)-{2(*S*)-[2(*R*)-amino-3-mercapto]-propylamino-3(*S*)-methyl}(pentyloxy-3-phenyl-propionylmethioninesulfone methyl ester (L-739, 749) has recently been reported to be an effective inhibitor of protein farnesyltransferase and of the growth of tumors arising from *ras*-transformed cells in nude mice [3]. Specific toxicities associated with treatment with this type of agent are as yet unknown.

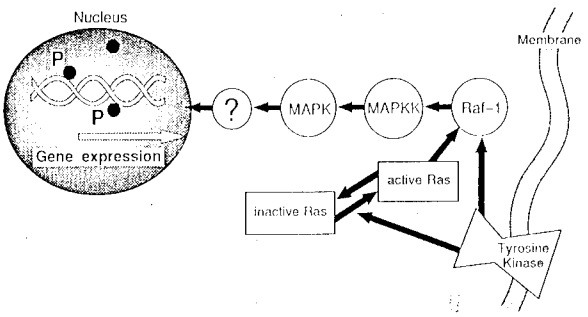


Fig. 1. The Ras signal transduction pathway. MAPK, mitogen-activated kinase; MAPKK, MAPK kinase; P, phosphate. Question mark indicates the role of other, as yet poorly defined, kinases.

2. Inhibitors of protein tyrosine kinases

The protein products of many proto-oncogenes are protein tyrosine kinases, and the aberrant expression of these enzymes is associated with some human tumors [4]. Activity of oncogenic tyrosine kinases is required to induce proliferation and to elicit and maintain a transformed cellular phenotype. Agents capable of interfering with this sequence of events are attractive targets for the treatment of proliferative diseases. There are 2 major types of tyrosine kinases, those linked to a growth factor receptor and those which are not. A variety of chemicals have been investigated in the search for small molecule inhibitors of this process and only a few are mentioned here. Prominent among them are the flavonoids, a group of oxygen-containing heterocyclic molecules. The plant product quercetin is shown here as the representative of this class of compound (Fig. 2A). It possesses properties of interest to re-

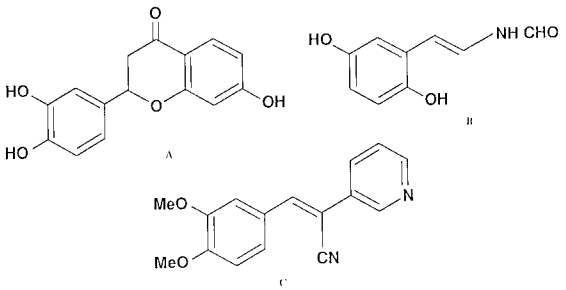


Fig. 2. Structures of inhibitors of protein tyrosine kinase. (A) Quercitin; (B) erbstatin; (C) a tyrphostin.

search workers in chemotherapy, as it is an anticarcinogen [5] and sensitizes human lung tumors grown in nude mice towards *cis*-platinum [6]. Co-administration with platinum-type drugs may be a plausible therapeutic application for this agent. Recently quercetin underwent phase I clinical evaluation in cancer patients at the Queen Elizabeth Hospital in Birmingham (UK). Reversible nephrotoxicity was the dose-limiting toxicity and occurred in all patients who received the highest dose (1700 mg/m^2; Ferry, Kerr et al., unpublished). We have investigated the risk of genotoxicity associated with combining quercetin with *cis*-platinum. In the bacterial mutagenicity test using 3 *Salmonella* strains mutagenicity was not increased above that elicited by the individual components on their own. However in the unscheduled DNA synthesis assay in rat hepatocytes quercetin decreased the *cis*-platinum-induced elevation in nuclear grain count (Cross, Chipman, Ferry and Gescher, unpublished). It remains to be shown whether quercetin elicits this effect via decreasing *cis*-platinum-DNA interaction or interfering with DNA repair activity.

Tyrosine kinase inhibitors of the flavonoid class do not discriminate between enzyme types, and they also inhibit other threonine and serine kinases. Another group of inhibitors possess the styrene moiety within their structure. The microbial product erbstatin (Fig. 2B) is particularly potent against the tyrosine kinase associated with the EGF receptor [8]. Tyrphostins comprise the largest and best studied group of tyrosine kinase inhibitors [9]. They are characterized by the hydroxy-cinnamonitrile pharmacophore. Structure C in Fig. 2 is an example of a 'second generation' tyrphostin which has shown encouraging antitumor activity in nude mice [10].

3. Modulators of protein kinase C (PKC)

PKC is a ubiquitous enzyme family which is a central constituent of a cascade of events triggered by mitogens and hormones via receptors on the cell surface (Fig. 3). It is involved in a plethora of physiological responses, including mitogenesis. Receptor-ligand interactions induce the activation of phospholipases, which catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate. This hydrolysis generates the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which mobilizes Ca^{2+} from intracellular stores [11]. DAG activates PKC by increasing its affinity for Ca^{2+}. Consequently IP3-mediated Ca^{2+} mobilization and PKC activation are often synergistic in their effects. There are alternative routes of DAG production, and its action is transient. Sustained levels of DAG and thus more protracted activation of PKC are believed to be mediated by hydrolysis of phosphatidylcholine. The latter pathway has therefore been strongly implicated in mitogenic events. The realization that PKC is an important arbiter of the regulation of cell proliferation, differentiation and tumor promotion became clear in the wake of the discovery that tumor-promoting phorbol esters can substitute for DAG in the activation of the enzyme [12]. One of the many fascinating features of PKC is the fact that it is a family of at least 12

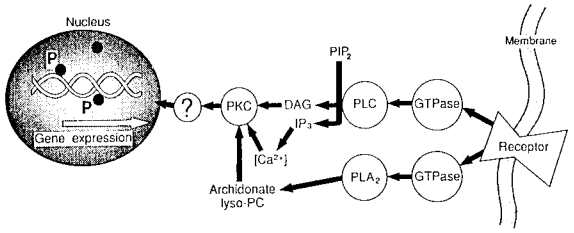
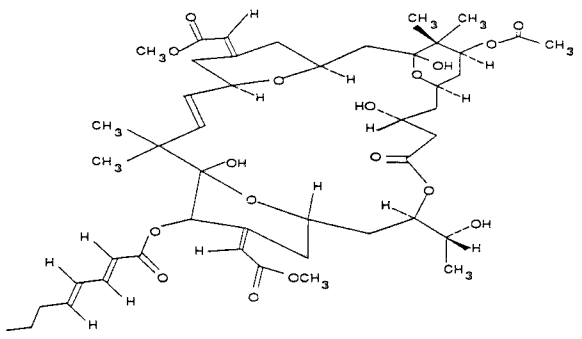


Fig. 3. Signal transduction pathway involving PKC. DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate: PC, phosphatidylcholine; PIP2, phosphatidylinositol 4,5-bisphosphate; PLA2, phospholipase A2; PLC, phospholipase C. Question mark indicates the role of other, as yet poorly defined, kinases.

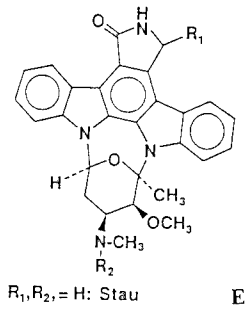
distinct isoenzymes. Each enzyme can be placed in 1 of 3 groups, characterized by differential requirements for activation by Ca^{2+} and phospholipid and responsiveness to DAG and phorbol esters. Many putative substrates of PKC have been described, but to date only a few have been characterized as being of physiological importance. On phosphorylation they precipitate a cascade of further phosphorylations, eventually converging on, and regulating the activity of $p34^{cdc2}$, a nuclear protein kinase, which steers the cells through mitosis.

Why is PKC of interest to cancer pharmacologists? Its function is altered in some neoplasias, leading to uncontrolled proliferation. Such dysfunction could be the consequence of a fault in the enzyme itself or of constitutive activation or repression of the PKC signalling pathway by aberrant components upstream or downstream from PKC. Some experiments suggest that over- or underexpression of PKC plays a role in tumorigenesis, depending on tissue type. For example, PKC activity is often elevated in human breast tumors when compared to adjacent normal tissue, and in human malignant glioma compared to non-neoplastic tissue. PKC isoforms-α, -βI, -γ or -ε mediate various growth abnormalities indicative of incomplete transformation and tumorigenicity [13]. In contrast, in colon tissue PKC has been implicated as possessing tumor-suppressive activity, where its loss leads to the development of a carcinoma [14]. Clearly the consequences of altered PKC are highly dependent on the nature of the cell type in which these changes occur, a conclusion which has to be considered carefully when attempts are made to unravel the role of PKC in transformation. PKC has also been proposed to influence the metastatic spread of tumors and the development of resistance against anticancer drugs.

A number of PKC modulators are currently undergoing clinical evaluation as anticancer drugs, or are in preparation for clinical trial. The fact that not only inhibitors but also an activator are among them seems paradoxical, but its reflects the multi-faceted and complicated nature of the role of PKC in cellular processes. Bryostatin 1 (Fig. 4D), a particularly interesting compound, is, like the phorbol esters, a potent PKC activator. It is one of 17 macrocyclic lactone derivatives which were isolated from marine bryozoans and characterized in the early 1970s by G.R. Pettit and coworkers at Arizona State University. Recent clinical trials of bryostatin 1 have been initiated on the bases of its broad-spectrum antineoplastic activity in rodent tumor models in vivo, including the P388 lymphocytic leukaemia, M5076 ovarian sarcoma, B16 melanoma and L10A lymphoma [15]. Bryostatins are also immunomodulators; they activate T cells, augment neutrophil and monocyte-mediated cytotoxicity and stimulate bone marrow progenitor cells. The paradoxical nature of their biological activity is puzzling. In many cell lines byrostatins are not only agonistic with tumor-promoting phorbol esters, but at certain concentrations also able to antagonize biochemical responses elicited by themselves or by phorbol



D



R_1, R_2 = H: Stau E
R_1 = OH, R_2 = H: UCN F
R_1 = H, R_2 = benzoyl: CGP G

Fig. 4. Structures of modulators of PKC. (D) Bryostatin 1; (E) staurosporine; (F) UCN-01; (G) CGP 41251.

esters. Relevant to the prospective use of bryostatin 1 in the clinic is the finding that, unlike phorbol esters, it lacks tumor-promoting properties in the Sencar mouse model and actually inhibits phorbol ester-induced tumor promotion [16]. The reasons why bryostatins behave differently from phorbol esters despite similar effects on PKC are unclear. Two phase 1 clinical trials of byrostatin 1 in the UK [17,18] have shown that, when administered as a bolus infusion via the i.v. route for 1 h once a week for 3 weeks, the observed maximum tolerated dose, between 25 and 50 $\mu g/m^{-2}$, is extremely low. The major toxicity observed was an impervious myalgia, but there was no significant myelosuppression, apart from a small and transient fall in platelet count. Although myalgia is an unusual toxicity for an antineoplastic agent, it should not be surprising to find that an investigational drug with a novel mechanism of action displays exotic adverse effects.

PKC inhibitors can be divided into 2 broad classes: those which inhibit the activity of the catalytic domain, competing with the phosphate group donor ATP or the substrate, and those which target the regulatory domain of the enzyme, competing with cofactors. The microbial product staurosporine (Fig. 4E) is the prototypical PKC inhibitor. It interacts with the kinase domain of PKC with high potency, but because of the ATP-competitive nature of its inhibitory action it also impedes a range of other kinases, such as $pp60^{v\text{-}src}$ tyrosine kinase, with similar potency. Drug discovery efforts by several pharmaceutical companies have yielded staurosporine analogs which appear to be more selective for PKC whilst retaining high enzyme-inhibitory potency. Three notable examples are the hydroxylated staurosporine derivative UCN-01 (Fig. 4F) developed by Kyowa Hakko in Japan, the *N*-benzoyl derivative CGP 41251 (Fig. 4G) from Ciba Geigy in Switzerland, and a series of bisindolylmaleimides designed by Roche UK and Glaxo France. UCN-01 and CGP 41251 possess activity against human tumors in mice and are about to enter clinical trial.

The mechanisms by which staurosporine and its analogs mediate their cytostatic effects are unclear. Recent work in our laboratory has attempted to explore these mechanisms. We compared staurosporine and 4 cogeners, UCN-01, CGP 41251 and the bisindolylmaleimides RO 31 8220 and GF 109203X, in A549 and MCF-7 cells. We reported that RO 31 8220 and GF 109203X are much less potent inhibitors of the growth of these cells than staurosporine, UCN-01 and CGP 41251, whereas all 5 compounds were strong inhibitors of the PKC contained in these cells, with IC_{50} values of below 100 nM [19]. More recently MCF-7 cells with acquired resistance to doxorubicin (MCF-7/Adr) were found to be resistant towards the growth-arresting properties of RO 31 8220 and UCN-01, with resistance ratios of 12.6 and 7.0, respectively (Budworth, Malkhandi, Ferry, Gant and Gescher, submitted). The ratios for GF 109203X, staurosporine and CGP 41251 were 1.2, 2.0 and 2.9, respectively. Reserpine, which modifies multidrug resistance mediated by P-glycoprotein (Pgp), reversed resistance against RO 31 8220 and UCN-01. Staurosporine and CGP 41251 at 10 and 20 nM, respectively, decreased efflux of the Pgp probe rhodamine 123 from MCF-7/Adr cells, whereas RO 31 8220 and GF 109203X at 640 nM were inactive. CGP 41251 was the most effective and GF 109203X the least effective inhibitor of equilibrium binding of [^{3}H]vinblastine to Pgp of MCF-7/Adr cells. The results of these studies proffer the following conclusions:

(i) PKC is unlikely to play a direct role in the growth arrest mediated by these agents.

(ii) The structure-activity relationships among the staurosporine analogs which govern cytostatic potency, affinity for Pgp and susceptibility towards Pgp-mediated substrate transport are complex and do not correlate with ability to inhibit PKC.

(iii) The kinase inhibitors appear to affect Pgp directly and not via inhibition of PKC-modulated Pgp phosphorylation.

A number of drugs in clinical use, such as chlorpromazine, adriamycin and suramin, are moderately effective PKC inhibitors. One of the most potent of them is tamoxifen. It inhibits PKC at the regulatory domain at clinically effec-

tive drug concentration. This inhibition might contribute, at least in part, to the anti-breast cancer activity of tamoxifen.

4. Conclusion

The recent remarkable progress in the understanding of signalling pathways has generated considerable excitement among researchers in the area of anticancer drug discovery. One has to bear in mind though that the degree of redundancy which exists within kinase pathways might confound the usefulness of selective modulators as drugs. For example, a particular PKC isoenzyme might substitute for another one when it is selectively inhibited. A great deal remains to be discovered about the way in which antiproliferative modulators of signal transduction interact in vivo with the plethora of kinases which drive cell growth. Novel toxicities may confound therapeutic gain. Results of studies addressing these issues should eventually help to exploit our ability to influence cellular signalling to the benefit of patients. Undoubtedly the clinical evaluation of agents as intriguing as quercetin, bryostatin 1 and CGP 41251 will contribute to unravelling the enigma.

Acknowledgements

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Toxicology Letters

Anticancer drug toxicity via cytokine production: the hydroxyurea paradigm

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Abstract

Our previous observations on the toxic effects of hydroxyurea (HU) in adrenalectomized (ADX) rats prompted us to suggest that these effects might be mediated by an increased synthesis of proinflammatory cytokines. This study was conducted to determine whether HU stimulates cytokine gene expression in vivo. The polymerase chain reaction (PCR) technique was used to assess levels of mRNA for interleukin-1α (IL-1α), tumor necrosis factor (TNF) and interleukin-4 (IL-4) in spleen cells from intact and ADX rats treated with HU or vehicle. In ADX rats, expression of proinflammatory-cytokine mRNA was markedly increased by HU, but no expression of these genes was seen in intact animals after treatment. In the latter rats, cytokine-gene expression seemed to be down-regulated by HU-induced elevations in plasma corticosterone levels, since IL-1α and TNF transcripts could be detected only after corticosterone levels had returned to normal (24 h after treatment). Interestingly, IL-4 mRNA could not be detected in either treated or untreated ADX rats, indicating that expression of this gene is closely related to circulating levels of corticosterone. These findings strongly suggest that the increased toxicity displayed by HU in ADX animals is mediated by stimulation of cytokine synthesis in vivo.

Keywords: Hydroxyurea; Interleukin-1; Interleukin-4; Tumor necrosis factor; Adrenal glands (rat)

1. Introduction

In a previous study [1] we found that single or repeated administration of the anticancer drug, hydroxyurea (HU) causes dose-dependent adrenal activation in rats. The increased secretion of corticosterone (B) that results appeared to protect the animals from the toxic effects of this drug, which were, in fact, dramatically enhanced in adrenalectomized (ADX) or hypophysectomized rats. Treatment of ablated rats with 300–800 mg/kg/day p.o. for 5 days resulted in up to 100% mortality (caused by severe cardiovascular collapse – unpublished observation).

The proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), also activate the hypothalamo-pituitary-adrenal axis without inducing tolerance. In ADX animals, the toxic effects of these cytokines are much more severe and include an endotoxic-like shock [2] that resembles that of ablated rats treated with HU. These similarities led us to

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hypothesize that HU toxicity in ADX animals might be mediated by an increase in the synthesis of proinflammatory cytokines [3]. HU is known to potentiate the stimulatory effect of bacterial lipopolysaccharide on IL-1 production by myelomonocytic cells in vitro [4], but the effect of this drug on in vivo cytokine synthesis has never been investigated.

In the present study, intact and ADX rats were treated with HU, mRNA was extracted from spleen cells 2 and 24 h after treatment, and message levels for IL-1α, TNF and interleukin-4 (IL-4) were evaluated using the polymerase chain reaction (PCR) technique. Plasma B levels were also measured in parallel with a specific radioimmunoassay.

2. Materials and methods

2.1. Experimental procedures

Male Wistar rats weighing 200 g were acclimatized for a period of 7 days in a room maintained at a temperature of 23 ± 1.5°C with a relative humidity of $65 \pm 2\%$. The animals were exposed to 12 h light (06:00–18:00 h) followed by 12 h dark and had free access to food pellets and water. Lumbar adrenalectomy, as described by Pomeau-Delille [5], was performed on some of the rats. These animals were given normal saline instead of water and were used 1 week after surgery.

On the day of the experiment, intact and ADX rats were treated with HU 800 mg/kg via gastric gavage and decapitated 2 or 24 h later. Trunk blood was collected for plasma B assays. The spleens were rapidly dissected and kept in ice-cold Earle's Balanced Salt Solution (EBSS, Sera-Lab Ltd, Crawley Down, Sussex, UK) containing penicillin G sodium 15 μg/ml and streptomycin sulphate 25 μg/ml (both from Sigma Chemical Co., St. Louis, MO). The spleens were processed for mRNA extraction on the day of the experiment.

2.2. RNA preparation and detection of cytokine transcripts by PCR

These procedures have been previously described in detail [6,7]. Briefly, 5×10^6 spleen cells were subjected to RNA extraction by the guanidium thiocyanate-phenol-chloroform procedure. Purified total RNA was incubated with 0.5 μg oligo(dT) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 h at 42°C after adding 20 U RNAase inhibitors (Boehringer-Mannheim Italia Spa, Milan, Italy), 1.5 mM deoxynucleoside triphosphates, 7.5 U avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) and reverse transcriptase buffer (50 mM Tris–HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl and 10 mM DTT, final concentrations) in a final volume of 20 μl. The cDNA was diluted to a total volume of 75 μl with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and frozen at −20°C until use.

Amplification of synthesized DNA was carried out using IL-1α (5′-ATG GCC AAA GTT CCT GAC TTG TTT-3′ and 5′-C CTT CAG CAA CAC AGG CTT GTC T-3′), TNFα (5′-ATG AGC ACG GAA AGC ATG ATC CGA-3′ and 5′-CC AAA GTA GAC CTG CCC GGA CTC-3′), IL-4 (5′-ATG GGT CTC AAC CCC CAC CTT GC-3′ and 5′-GAC TAA CTC AGC CTC CAC GAA GTA-3′), or β-actin specific 5′ sense and 3′ antisense primers. Briefly, 1–5 μl cDNA was added to a reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 3.0 mM $MgCl_2$, 0.01% gelatin, 0.2 deoxynucleoside triphosphates, 1 μM of each primer and 0.5 U AmpliTaq polymerase (Perkin-Elmer Corp., Hayward, CA). Each 20-μl sample was overlayed with 25 μl mineral oil (Sigma Chemical Co., St. Louis, MO) and incubated in a DNA Thermal Cycler 480 (Perkin-Elmer Corp.) for a total of 30 cycles: 1 min at 94°C, 1 min at 67°C or 60°C (β-actin), and 1 min at 72°C. The amplified DNA size, as compared to a positive control, was 625 bp for IL-1α, 692 bp for TNFα, 398 bp for IL-4, and 540 bp for β-actin. The β-actin primers were used as a control for both reverse transcription and the PCR reaction itself, and also for comparing the amount of products from samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was

repeated at least twice for at least 2 separately prepared cDNA samples for each experiment. Data are representative of at least 2 different experiments. Under the employed conditions, control samples from naive rats showed, as a rule, no background cytokine mRNA levels, so that the magnitude of the response to treatment could be easily demonstrated.

2.3. Radioimmunoassay for plasma corticosterone

This technique has been described in detail elsewhere [1].

3. Results

3.1. Proinflammatory cytokines

Weak expression of IL-1α and TNF mRNAs was detected in specimens from intact rats treated with vehicle. HU treatment of intact animals completely abolished this expression and also caused a significant increase in plasma B levels, as compared to those found in untreated controls. Plasma B levels and transcripts of both cytokines had returned to control levels 24 h after administration of HU (Figs. 1 and 2).

Plasma B was almost undetectable in all of the ADX animals with the exception of one whose adrenalectomy had probably been incomplete. Ablated animals treated with vehicle presented basal expression of mRNA for IL-1α and TNF. In contrast to that observed in intact animals, HU treatment of these rats markedly enhanced the expression of TNF mRNA and, to a lesser extent, that for IL-1α. The effect of the drug on cytokine gene expression in ADX rats was already significant 2 h after oral treatment, and was no longer evident 24 h later (Figs. 1 and 2).

3.2. IL-4

While the pattern of expression of mRNA for IL-1α and TNF appeared to be related to both HU treatment and plasma B levels, that for IL-4 seemed to be directly related only to the latter parameter. Basal IL-4 mRNA expression was detected in intact control rats, and transcript

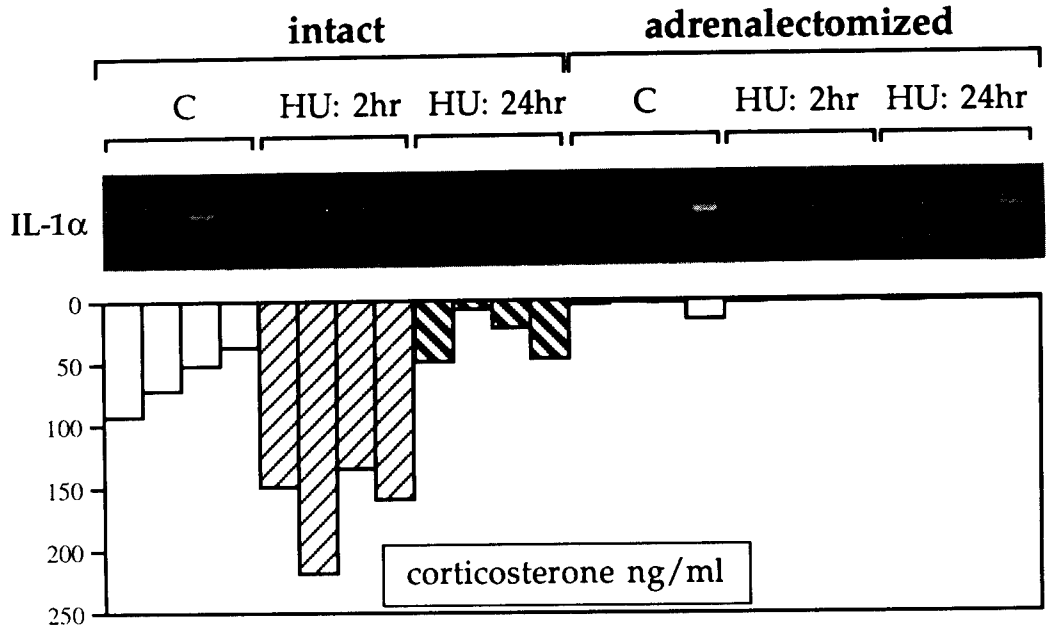


Fig. 1. *Upper panel*: IL-1α mRNA expression in spleen cells from HU-treated rats, as revealed by PCR (normalized to β-actin, not shown). RNA was isolated from intact or ADX rats treated with vehicle (C) or HU 2 or 24 h earlier, and the resulting cDNA was used in the PCR with IL-1α-specific primers. After amplification, 10 μl of the reaction mix was removed, analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. *Lower panel*: plasma corticosterone levels found in the same rats. Each experimental group consisted of 4 rats assayed individually.

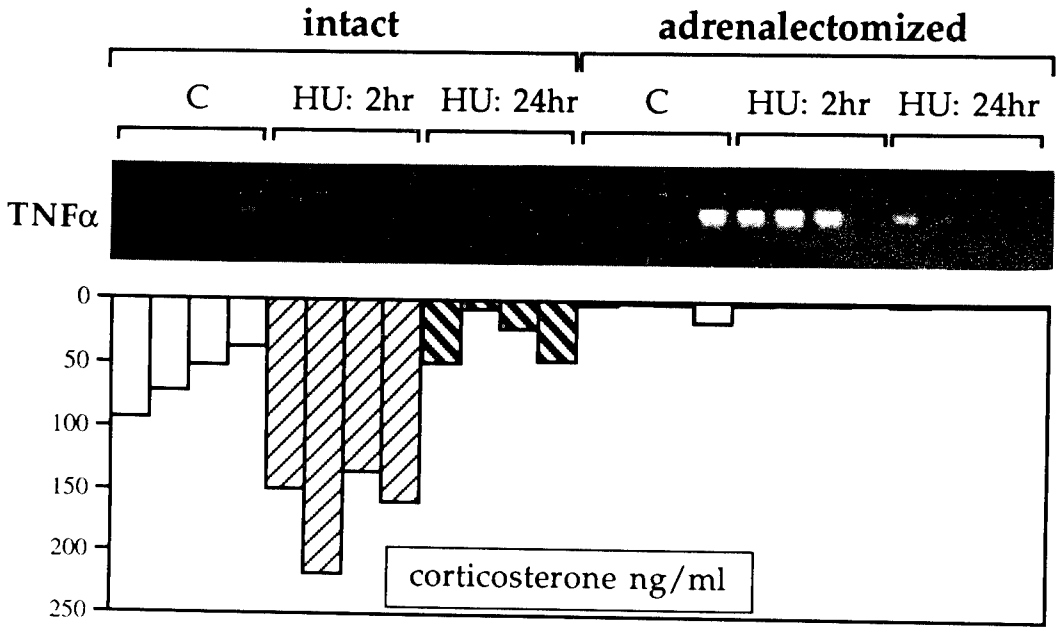


Fig. 2. *Upper panel*: TNF mRNA expression in spleen cells from HU-treated rats revealed by PCR. *Lower panel*: plasma corticosterone levels. See also legend to Fig. 1.

levels (as well as plasma B levels) were significantly increased by HU. No IL-4 message was expressed in any of the ADX rats (with the exception of the one with detectable plasma B levels described above), regardless of whether or not they had been treated with HU (Fig. 3).

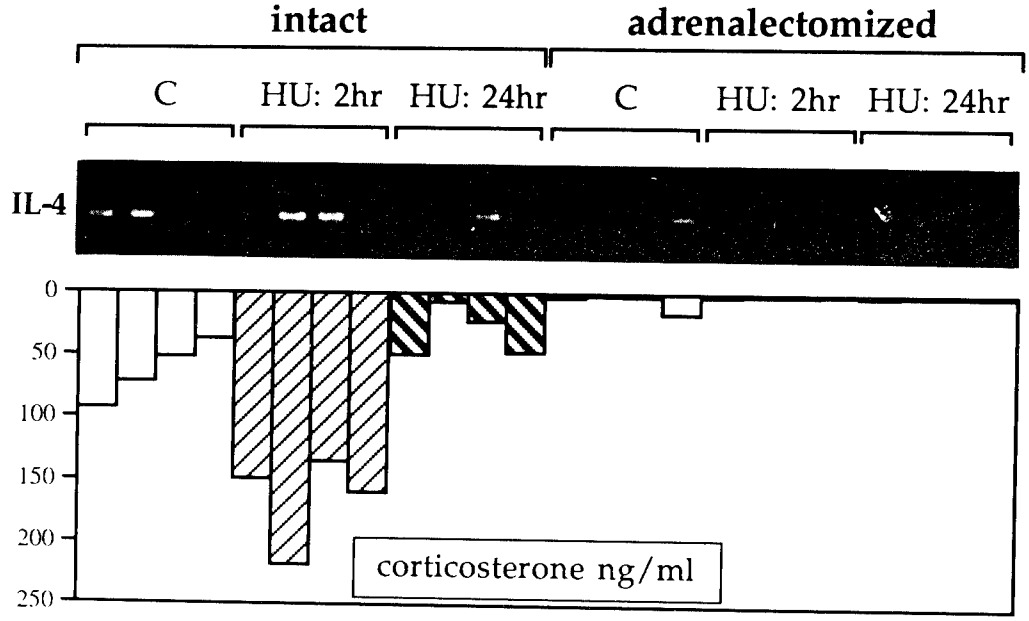


Fig. 3. *Upper panel*: IL-4 mRNA expression in spleen cells from HU-treated rats revealed by PCR. *Lower panel*: plasma corticosterone levels. See also legend to Fig. 1.

4. Discussion

Our data show that, in the absence of endogenous glucocorticoids, HU stimulates the in vivo expression of genes coding the proinflammatory cytokines IL-1α and TNF. Although these findings must be confirmed by the demonstration of increased cytokine synthesis, they strongly suggest that the increased HU toxicity observed in ADX animals may be mediated by proinflammatory cytokines.

The mechanisms by which this drug increases gene expression for these cytokines is still unclear. The primary effect of HU on cell metabolism is an inhibition of ribonucleoside reductase, which leads to a reduction in DNA synthesis and, as a result, changes in cell-cycle kinetics that might account for the altered gene expression we observed. This mechanism does not, however, explain why only proinflammatory cytokines are stimulated, while others (e.g. IL-4) are unaffected by the drug.

A highly interesting and unprecedented observation that emerged from this study (although not directly related to the effects of HU) was that of the in vivo induction of IL-4 mRNA by glucocorticoids. IL-4 is known to exert antiinflammatory effects that are mediated, at least in part, by the inhibition of gene expression and release of proinflammatory cytokines [8,9]. If our findings can be confirmed in specific studies, another element might be added to the long list of mechanisms through which glucocorticoids exert their antiinflammatory effects.

In conclusion, the results of the present study strengthen our hypothesis that the enhanced toxicity of HU seen in ADX rats is mediated by proinflammatory cytokines. Adrenal steroids appear to protect intact animals against these effects in at least 2 ways: (1) by direct inhibition of IL-1α and TNF gene expression; and (2) by induction of the antiinflammatory cytokine IL-4.

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Chemoprotection against cancer by Phase 2 enzyme induction

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Abstract

Mammalian cells have evolved elaborate mechanisms for protection against the toxic and neoplastic effects of electrophilic metabolites of carcinogens and reactive oxygen species. Phase 2 enzymes (e.g. glutathione transferase, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases) and high intracellular levels of glutathione play a prominent role in providing such protection. Phase 2 enzymes are transcriptionally induced by low concentrations of a wide variety of chemical agents and such induction blocks chemical carcinogenesis. The inducers belong to many chemical classes including phenolic antioxidants, Michael reaction acceptors, isothiocyanates, 1,2-dithiole-3-thiones, trivalent arsenicals, $HgCl_2$ and organomercurials, hydroperoxides, and vicinal dimercaptans. Induction by all classes of inducers involves the antioxidant/electrophile response element (ARE/EpRE). Inducers are widely, but unequally, distributed among edible plants. Search for such inducer activity in broccoli led to the isolation of sulforaphane, an isothiocyanate that is a very potent Phase 2 enzyme inducer and blocks mammary tumor formation in rats.

Keywords: Glutathione transferases; Quinone reductase; Vegetables; Antioxidant response element (ARE); Electrophile response element (EpRE)

1. Introduction

The initiation of many tumors results from damage to DNA by electrophilic carcinogen metabolites, or by reactive oxygen species that arise during carcinogen metabolism or endogenous cellular processes. Mammalian cells have evolved multiple and elaborate mechanisms for protection against such toxic insults. Phase 2 enzymes (e.g. glutathione transferases (GST), NAD(P)H:quinone reductase (QR), epoxide hydrolase, glucuronosyltransferases, aldehyde reductase, and others) and high cellular levels of glutathione are the primary lines of defense against these reactive chemical species. These protective mechanisms disarm and facilitate the disposal of reactive electrophiles and oxygen species. Much recent evidence indicates that elevation of Phase 2 enzymes and of glutathione levels by inducers results in protection against chemical carcinogens. Indeed, modulation of the metabolism of carcinogens is one of the most effective and well-established strategies for protecting animals and their cells against the toxic and neoplastic effects of carcinogens.

Phase 2 enzymes are transcriptionally reg-

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ulated in animal cells by low concentrations of a wide variety of chemical agents, many of which are already present in the human diet [1–3]. Consequently, specific modification of the human diet to increase the consumption of phytochemicals that induce Phase 2 enzymes is an attractive, safe, and promising strategy for decreasing the risk of developing cancer. Implementation of this strategy requires: (a) identification of effective inducers and determination of their inducer potencies; (b) elucidation of the chemistry and molecular mechanisms of action of inducers; (c) identification of edible plants rich in inducer activity; (d) demonstration that such plants can raise Phase 2 enzymes when administered to animals and to humans; (e) trials in humans with short-term surrogate biomarkers for the protected state; and (f) ultimate demonstration of risk reduction in human populations at high risk of developing cancer. This brief account reviews progress in achieving these objectives.

2. Detection and identification of Phase 2 enzyme inducers and measurement of their potencies

Since Phase 2 enzymes are generally induced coordinately in many tissues and in cells in culture, we have selected a single enzyme as a marker for induction. Quinone reductase (QR) is a convenient representative enzyme because it is widely distributed in mammalian tissues, is easily measured, and shows a large inducer response (as much as 10- to 12-fold in some tissues) [4]. A highly suitable and robust cell line for studying induction of this enzyme is the Hepa 1c1c7 murine hepatoma line [1]. Measurement of QR activity directly (by a coupled tetrazolium dye assay) on digitonin extracts of cells grown in 96-well microtiter plates and exposed to serial dilutions of the inducer (a single chemical compound, a mixture, or a plant extract) provides an accurate assessment of inducer activity [5,6]. The specific activity of the enzyme can then be obtained by relating the activity to cell mass or protein concentration. A convenient index of inducer potency is the concentration required to double (CD) the specific activity of QR. Inducers vary enormously in potency, with CD values ranging from low nanomolar to high millimolar concentrations.

The availability of mutant Hepa 1c1c7 cells that are defective in cytochrome P-450 activity or in *A*ryl *h*ydrocarbon (*Ah*) receptor function provides the means for distinguishing *monofunctional* inducers (that elevate Phase 2 enzymes selectively) from *bifunctional* inducers (that upregulate both Phase 1 and Phase 2 enzymes) [7,8]. This distinction is important because some cytochromes P-450 (e.g. 1A1 and 1A2) are involved in activation of carcinogens, whereas Phase 2 enzymes mostly catalyze detoxication reactions. Monofunctional inducers are therefore preferred as agents for achieving chemoprotection in humans.

3. The chemistry of inducers

The development of detailed understanding of the chemical requirements for inducer activity has been a continuing effort in our laboratory since the demonstration that phenolic antioxidants exerted their major chemoprotective activity by virtue of the induction of glutathione transferases and other Phase 2 enzymes [9,10]. The first insight into the chemistry of inducers was obtained from studies of structural analogues of BHA [11], a chemoprotective antioxidant that is widely used as a food additive. These studies pointed to *tert*-butylhydroquinone, a metabolite of BHA, as probably the active species responsible for the inducer activity of BHA.

Major information on the chemistry of inducers was obtained from studies of analogues of *tert*-butylhydroquinone in which the orientation of the diphenolic hydroxyl groups was changed from 1,4-diphenols to 1,2- or 1,3-diphenols. The results were clear-cut: only the 1,2-diphenols (catechols) or the 1,4-diphenols (hydroquinones) were inducers, whereas the 1,3-diphenols (resorcinols) were inactive, and the presence or absence of other ring substituents was relatively unimportant in specifying inducer activity [12].

These results clearly implicated redox lability in inducer function, since catechols and hydroquinones can be readily oxidized to the corresponding quinones, whereas resorcinols cannot undergo such conversions. These experiments did not, however, reveal whether the quinone products or the redox process itself (perhaps the reactive oxygen species generated) was responsible for induction. This issue was resolved by the finding that many highly electrophilic Michael reaction acceptors (olefins or acetylenes conjugated to electron-withdrawing groups) were inducers, and that their potency generally paralleled their reactivity in the Michael reaction [2]. Since quinones are excellent Michael reaction acceptors, the inducer activity of 1,2- and 1,4-diphenols is therefore dependent upon their oxidation to quinones.

The chemistry of inducers was subsequently greatly expanded [13,14] with the observation that, in addition to oxidizable diphenols (and corresponding phenylenediamines) and Michael reaction acceptors, the following classes of compounds are also efficient inducers: isothiocyanates, 1,2-dithiole-3-thiones, trivalent arsenicals, mercury(II) salts and organic mercurials, and hydroperoxides. These inducers share almost no structural similarities (in the sense of complementarity to a receptor), but are all electrophiles, capable of reacting with sulfhydryl groups. These findings suggested that the signaling of induction involves a primary interaction with a highly reactive sulfhydryl group, or possibly two vicinal sulfhydryl groups, since trivalent (but not pentavalent) arsenicals are excellent inducers. It was therefore somewhat surprising that several compounds carrying vicinal sulfhydryl groups (e.g. 2,3-dimercapto-1-propanol (BAL)), but not monothiols, were also efficient inducers [13,14]. Although these compounds are nucleophiles rather than electrophiles, they can, like all other inducers, modify sulfhydryl groups by redox reactions. We conclude that the following general properties characterize all known inducers: (a) most are electrophiles (including quinones and Michael reaction acceptors); (b) all react with sulfhydryl groups by virtue of their electrophilicity, or by participating in redox reactions; and (c) most inducers are substrates for glutathione transferases [15].

4. Presence of Phase 2 enzyme inducers in edible plants

The extensive evidence that increased consumption of fruit and vegetables is associated with reduced risk to developing cancer [16], naturally raised the issue whether at least some of these effects might be due to the presence of Phase 2 enzyme inducers in edible plants. When the inducer potencies of organic solvent extracts of a variety of commonly-consumed plants were measured, there were marked differences depending on genus, species, and even variety [6]. Cruciferous plants (e.g. broccoli, cabbage, cauliflower, kale) were particularly rich sources of inducer activity. We selected broccoli for detailed study because extracts tended to have high inducer activity and broccoli was already consumed in substantial quantities in the Western world. One cultivar of broccoli (SAGA) was particularly rich in inducer activity. Reverse phase HPLC and other forms of chromatography showed that the majority of the inducer activity of Saga broccoli was attributable to a single compound, an isothiocyanate: sulforaphane (CH_3-S(O)-$(CH_2)_4$-N=C=S) [17]. Sulforaphane, which had been previously isolated from cabbage [18] and had also been synthesized [19], was found to be an exceedingly potent QR inducer in murine hepatoma cells; indeed it is the most potent naturally occurring inducer so far identified. When fed to mice, sulforaphane induced both QR and glutathione transferases in several tissues [17].

Analogues of sulforaphane that differ in the state of oxidation of the methylthio group and the length of the methylene bridge, i.e. $CH_3S(O)_m$ $(CH_2)_n$N=C=S, where $m = 0$, 1, or 2 and $n = 3$, 4 or 5, were prepared and tested for inducer activity in murine hepatoma cells [17]. Sulforaphane was the most potent inducer. The sulfoxides and the sulfones were more potent than the sulfides, and the compounds with four

or five methylene groups were more potent than those with only three methylene groups.

The methyl sulfinyl function of sulforaphane was very important for inducer activity since n-hexyl-N=C=S was a much weaker inducer than sulforaphane [20]. Interestingly, the methyl sulfinyl group (CH_3SO-) could be replaced by an acetyl group (CH_3CO-) without changing the inducer activity significantly. Consequently a number of cyclic analogues were designed in which the distance between the CH_3CO- and the -NCS groups was varied. Among the most potent inducers were certain acetylnorbornyl-NCS analogues, some of which were comparable in inducer potency to sulforaphane [20].

Although the majority of the inducer activity of extracts of SAGA broccoli was attributable to sulforaphane, we have shown recently that such extracts also contain lesser quantities of erucin (the sulfide analogue). Since erucin has only about one-sixth the inducer potency of sulforaphane, it makes only a minor contribution to the total inducer activity of SAGA broccoli extracts. In connection with our conclusion that sulforaphane is the principal Phase 2 enzyme inducer of SAGA broccoli extracts, we now realize that the conditions of isolation of sulforaphane involved the preparation of aqueous homogenates of broccoli that were then lyophilized [17]. More recent experiments indicate that these conditions were favorable for hydrolysis of glucoraphanin (the glucosinolate precursor of sulforaphane) by the coexisting thioglucosidase, myrosinase. It is therefore very likely that in the intact plant a significant proportion of the isolated sulforaphane exists as its glucosinolate.

5. Antitumor effects of sulforaphane

Sulforaphane and its norbornyl-NCS analogues were tested in the single dose DMBA (7,12-dimethylbenzanthracene) mammary tumor model in Sprague–Dawley rats [21]. The chemoprotectors were administered by gavage for 3 days before the DMBA, on the day of carcinogen treatment, and on the following day. Under these circumstances, there was a substantial, dose-dependent reduction in the incidence of mammary tumors that developed. In addition, there was a reduction in both the multiplicity (number of tumors per rat) and the size of the tumors, and tumor appearance was delayed. Sulforaphane and one of the acetylnorbornyl isothiocyanates were of similar potencies in their ability to block tumor development.

The observation of antitumor effects of sulforaphane and its analogues was not altogether surprising, because such properties had already been recognized in other isothiocyanates [22]. However, the finding of the high inducer and anticarcinogenic properties of sulforaphane encouraged us to undertake a systematic search for anticarcinogenic enzyme inducers in edible plants. The results confirmed the validity of the strategy of searching for naturally-occurring chemoprotectors in plants (and designing analogues) based on monitoring inducer potency. We are aware of only three prior examples of this approach: the prediction of the tumor blocking activity of a 1,2-dithiole-3-thione (oltipraz) [23,24], isolation of two terpenoids from green coffee beans [25]; and the demonstration of the blocking activity of the Michael acceptor dimethyl fumarate on hepatic tumor formation in A^{vy}/A mice (Y. Zhang and P. Talalay, unpublished observations).

6. Molecular mechanisms of the regulation of Phase 2 enzyme induction

The rational development of more effective chemoprotective Phase 2 enzyme inducers would be greatly facilitated by a detailed understanding of the molecular mechanisms underlying the regulation of these enzymes. As noted above, the inducers belong to at least eight different chemical families that have few common characteristics apart from their electrophilic reactivity and their ability to interact with sulfhydryl groups either by nucleophilic substitution or oxidoreductions. The proposal that a primary covalent interaction with sulfhydryl groups of a 'target' protein generates the signal for induction is supported not only by the propensity of all inducers to react with

sulfhydryl groups, but also by the finding that inducer potency is related to avidity for sulfhydryl groups. This is illustrated by the general correlation between inducer potency and the reactivity of inducers in the Michael reaction [2]. Furthermore, inducer potency of a series of heavy metals correlates with their affinity for sulfhydryl groups, i.e. $Hg^{2+} > Cd^{2+} > Zn^{2+}$ [26]. The exceptionally high inducer potency of trivalent arsenicals strongly suggests that vicinal sulfhydryl groups that can form cyclic thioarsenites may be involved in the inducer signalling. If we postulate that all inducers must interact with an intracellular 'target protein' in order to initiate the events resulting in enhanced transcription, it seems likely that the cognate sulfhydryl group(s) on this protein must be extremely reactive in comparison to other sulfhydryl groups, otherwise it would be difficult to envisage how the inducers could evade reaction with the very high concentrations of glutathione that prevail in the cytosol (3–8 mM). However, if, as is entirely possible, the ultimate inducing species were the glutathione conjugates of the inducers, these might then be transported to the target protein and there undergo cleavage by the more reactive vicinal sulfhydryl groups of the target protein.

Much more specific information is available on the precise regulatory regions of the genes that are transcriptionally activated by exposure to inducers. Deletion analyses of the upstream regions of the glutathione transferase Ya gene of rat and mouse liver have identified nearly identical 41-bp enhancer regions that respond to the aforementioned inducers [27–31]. When these DNA segments were inserted into plasmids designed for heterologous gene expression, and the resulting plasmids were transfected into hepatoma cells, responses to inducers were observed. The controlling elements have been further narrowed to regions termed the Antioxidant Response Element (ARE) [29] or the Electrophile Response Element (EpRE) [30,31], for which the consensus sequence G(or A)TGACNNNGC has been assigned. The mouse GST Ya gene contains two of these elements separated by five base pairs, whereas the rat GST Ya gene contains only one element. Similar sequences have also been identified in the upstream regions of the human and rat QR genes (reviewed in [32]).

In a recent study [13,14], we inserted the 41-bp enhancer elements derived from the 5′-upstream region of the mouse liver GST Ya gene together with its promoter into plasmids capable of expressing human growth hormone as reporter. These plasmids were transfected into hepatoma cells, and the concentration dependence of growth hormone expression was measured for 28 inducers belonging to all known chemical classes of inducers. The potencies of these compounds in driving heterologous gene expression were then compared with their potencies as inducers of QR in hepatoma cells. Although these potencies spanned nearly four orders of concentration magnitude, they were very similar in the two systems. Furthermore six structurally related compounds were inactive in both systems. These results led to the unequivocal conclusion that the transcriptional activation evoked by all classes of inducers could be fully accounted for by activation of the 41-bp element containing the ARE/EpRE.

The nature of the ARE/EpRE and its transcriptional binding factors has been controversial. The issue is whether the ARE/EpRE has the properties of phorbol ester responsive elements (TRE) (and is regulated by binding of AP-1 factors such as c-*fos* and c-*jun*) [31], or whether ARE/EpRE involves distinctly different mechanisms and transcription factors [33]. Although the consensus TRE sequence [TGAC(or G)TC(or A)A] bears some resemblance to the ARE/EpRE consensus sequence, it lacks the critical 3′-terminal GC bases. This question has been recently examined in several laboratories. In our experiments [34], the behavior to inducers of the above-described growth hormone reporter construct containing the 41-bp ARE/EpRE region derived from the mouse GST Ya gene was compared with the behavior of the same construct in which the ARE/EpRE elements were replaced by one or two consensus phorbol ester response elements (TRE). The wild-type sequence was highly activated by monofunctional inducers of various chemical types, but the con-

structs in which the ARE/EpRE sequences were mutated to TRE were not responsive. Furthermore, transfection of the ARE/EpRE reporter construct into F9 cells, which lack endogenous TRE binding proteins, produced substantial stimulation of growth hormone synthesis by the same inducers that also induced QR enzyme activity in untransfected F9 cells. These results strongly favor the view not only that the ARE/EpRE mediates the induction response to the various types of inducer, but also that this process is independent of phorbol ester responsive elements. This conclusion is supported by independent lines of evidence from other laboratories [35,36].

Acknowledgements

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Chemoprevention of breast cancer by tamoxifen: risks and opportunities

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Abstract

The antioestrogen tamoxifen is of proven efficacy in inhibiting the growth of oestrogen receptor positive breast cancers in women. In rats, long-term dosing leads to the development of hepatocellular tumours. Tamoxifen in this species is a genotoxic carcinogen. Metabolic activation by cytochrome P450-dependent enzymes leads to DNA damage detectable by ^{32}P-postlabelling. Factors important in the development of hepatocellular lesions were the nature and quantity of metabolism and promotion/progression of the DNA lesion by agents such as phenobarbital and cell proliferation. No evidence was found for tamoxifen-induced DNA damage in the livers of 7 women taking this drug therapeutically.

Keywords: Tamoxifen; DNA damage; Genotoxicity; Liver tumours; Metabolism

1. Introduction

Tamoxifen was developed in the late 1960s as an antioestrogen. This drug inhibits oestrogen-stimulated cell division but in some tissues it can also exhibit oestrogen-like activities. Tamoxifen is of proven efficacy in inhibiting the growth of oestrogen receptor positive breast cancers in women and is probably one of the safest chemotherapeutic drugs in common use. As adjuvant therapy for breast cancer it has few undesirable side effects. Tamoxifen also has a number of beneficial effects apart from its primary action on breast cancer cells. In treated women, it has a significant action in reducing serum cholesterol [1] and the incidence of fatal myocardial infarction [2]. In post-menopausal individuals it may also help to limit the development of osteoporosis (Fig. 1). Clinical trials are currently under way, primarily in the USA and UK, to test the use of tamoxifen as a chemopreventive agent for breast cancer in healthy women.

There are unquestionable benefits in the use of this drug for the treatment of women with breast

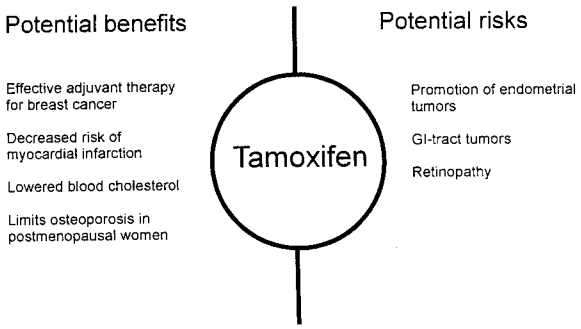


Fig. 1. Risk/benefit potential of tamoxifen.

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cancer. Its use as a chemopreventive agent in healthy women is not so clear-cut. Epidemiological evidence from women with breast cancer who have been treated with tamoxifen suggests long-term administration may result in a small increase in the incidence of endometrial [3] or GI tract tumours [4]. Further concerns about the potential safety of tamoxifen were raised when a number of independent laboratories found that long-term administration of tamoxifen to rats at high dose levels gave rise to hepatocellular carcinomas [5,6]. In these studies, there were no reports of tumours of the reproductive system or GI tract. There is also no epidemiological evidence to suggest that there is an increased risk of liver tumours in women taking this drug. However, experience with other carcinogens suggest that the organ or tissue affected is not necessarily the same across species.

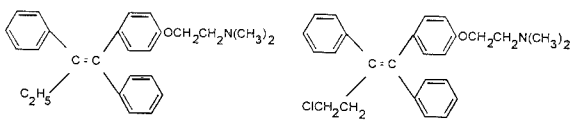
More evidence is required as to the mechanism of carcinogenic action of tamoxifen in experimental systems to inform better on risk-benefit analysis for women taking tamoxifen. Following the discovery that tamoxifen itself was not genotoxic but could be activated in the rat liver to give genotoxic intermediates [7], our aim has been to define the nature of the activating enzymes system, factors influencing DNA damage and the development of liver tumours. A principal objective of the study was to establish animal models which would permit identification of those factors which contribute to the development of hepatic tumours in the rat but not in the mouse. It might then be possible to determine whether they also operate in humans and if so, establish the potential risk of tamoxifen in women taking this drug.

2. Hepatic DNA damage caused by tamoxifen

Since all of the early in vitro tests for tamoxifen genotoxicity, such as the Ames *Samonella* assay, gave negative results, it was presumed, until a few years ago, that tamoxifen was working via an epigenetic mechanism, the hepatocarcinogenic effect in rats being in some way related to oestrogenic/antioestrogenic potency of this drug. Analogues such as toremifene (Fig. 2) have a similar antioestrogenic potency but do not give liver tumours in rats [6]. There is now good evidence that in the rat, tamoxifen is a genotoxic carcinogen. We have investigated the ability of tamoxifen and toremifene to induce DNA damage using the technique of ^{32}P-postlabelling. It was established that tamoxifen could cause adduct formation, even after a single dose, that was selective for the liver. In liver DNA, the degree of damage was dependent both on the dose and the length of exposure. Toremifene resulted only in trace levels of such damage [7]. Tamoxifen-induced ^{32}P-postlabelling was detected in mouse liver DNA but the level of adduct formation was about one-third that seen in rats.

Two features of the DNA lesions seen in rat liver following tamoxifen treatment may contribute to its hepatocarcinogenic effects. Firstly, following cessation of dosing, the adducts are repaired or eliminated very slowly with a half-life in the order of 3 months [8]. Secondly, with continuous exposure of rats to tamoxifen the extent of DNA damage continues to increase for many months. In adult rats, following 6–12 months exposure to tamoxifen, when there is rapid hepatocellular proliferation in hyperplastic nodules and tumours, the total extent of DNA damage decreases [9], probably as a result of dilution of the adducted DNA by preneoplastic cells.

There is strong evidence that cell proliferation plays an important role in the promotion and progression of DNA damage. In a study involving 3 strains of female rat given dietary tamoxifen corresponding approximately to 40 mg/kg/day, there were only small differences in its concentration and that of its major metabolites in the liver. Similarly, the extent of hepatic DNA damage, determined by ^{32}P-postlabelling at 6 months in all 3 strains were all similar [9]. At 3



Tamoxifen Toremifene

Fig. 2. Chemical structures of tamoxifen and toremifene.

months after dosing, using either conventional histochemical staining or the markers, γ-glutamyltranspeptidase or glutathione *S*-transferase P, the number of positive foci was about 10-fold higher in both Wistar and Lewis rats compared to the Fischer animals. There were also marked strain differences in the time to development of liver tumours with Wistar and Lewis animals being more susceptible. After 11 months, all of the Wistar and Lewis rats had liver carcinomas, while none was seen in the Fischer animals. Such carcinomas were seen in these animals only when they were killed at 20 months. A comparison of the extent of hepatic parenchymal cell division, relative to controls, showed that after 6 months tamoxifen exposure this was depressed in Fischer rats, in contrast to an increase in Wistar and in Lewis rats. It is concluded that the increase in cell proliferation is consistent with the promotion of foci to tumours and the subsequent progression of tumours in the latter 2 strains.

Promotion of liver DNA damage initiated by tamoxifen can be achieved by the use of phenobarbital. Wistar rats were dosed with tamoxifen for only 3 months and then returned to a basal diet. Tamoxifen is rapidly cleared from the body with a half-life of about 12 h. This drug could not be detected in the livers of rats 3 months after dosing even though liver DNA lesions detected by ^{32}P-postlabelling persisted. In a group a rats promoted with phenobarbital in the drinking water, the majority (12/14) consequently developed liver tumours. Even about one-third of those animals which received no additional promotion after the initial 3 months tamoxifen treatment went on to develop liver tumours in a lifetime study [8]. It was proposed that the persistence of the DNA adducts may account for the ability of phenobarbital to promote a high incidence of liver carcinomas after discontinuation of the tamoxifen dosing.

3. ^{32}P-Postlabelling of DNA from women taking tamoxifen therapeutically

Liver DNA samples obtained from 7 women receiving tamoxifen therapeutically or a 'control group' not receiving this drug were analyzed using ^{32}P-postlabelling. In both groups DNA damage was detected but the pattern of post-labelled spots was not the same as those detected in a tamoxifen-treated rat liver DNA. There was no difference in the level of DNA damage (18–80 adducts/10^8 nucleotides) between the 2 groups [8]. The marked difference between the level of hepatic DNA damage in rats which develop liver tumours (3000 adducts/10^8 nucleotides) and women suggests the hazard to humans is considerably less. Several factors may affect this finding. Firstly, in the study only 7 treated human livers were analyzed, compared with the very large numbers of women treated with this drug. Secondly, the individual susceptibility to tamoxifen treatment with respect to carcinogenicity is likely to be influenced by many factors. These may include genetic polymorphisms in Phase 1 or Phase II enzymes responsible for the activation and detoxication of tamoxifen and the balance between these pathways; the efficiency of DNA repair and the extent of cell proliferation. It cannot be excluded that a small number of women given tamoxifen, due to a combination of these factors, produce sufficient DNA damage to result in liver cancer nor can it be certain that tamoxifen does not damage DNA in other cell types of other organs.

4. Mechanisms of activation of tamoxifen to genotoxic intermediates

4.1. Effects of tamoxifen on hepatic drug metabolising systems

Tamoxifen administration to rats causes a 30–60-fold increase in the rate of metabolism of benzyloxyresorufin or pentoxyresorufin by liver microsomal preparations [10]. Smaller increases were seen in the 6β and 16α hydroxylation of testosterone as well as the oxidation of testosterone to androstenedione. Western blotting experiments showed a 2–3-fold increase in CYP2B1, CYP2B2 and CYP3A1 proteins in liver microsomal fractions. Tamoxifen acts as a weak inducer of these isoenzymic forms but the extent of induction is not nearly so marked as with 'classical inducers' such as phenobarbital or dexamethasone.

In rat liver microsomal systems in vitro,

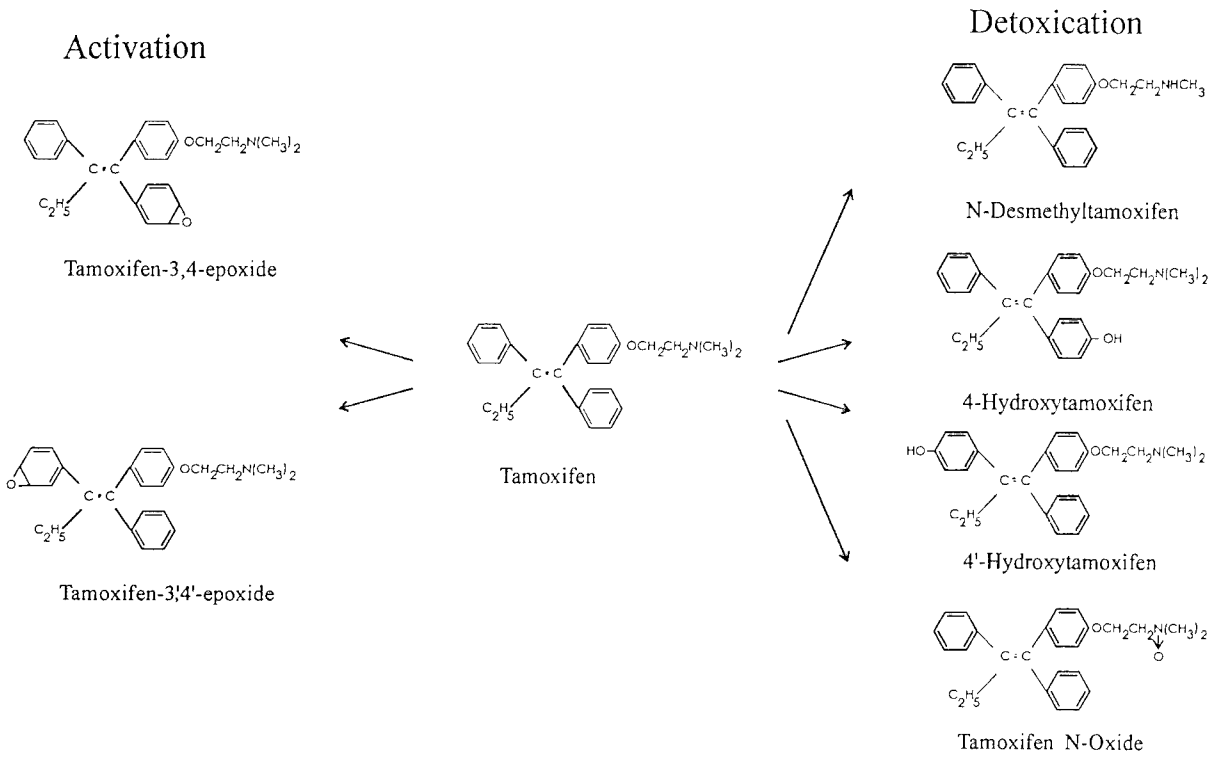


Fig. 3. Metabolic pathways for liver microsomal metabolism of tamoxifen.

tamoxifen is metabolised primarily by *N*-demethylation, *N*-oxidation and 4-hydroxylation (Fig. 3). These are Phase I detoxication reactions. Pretreatment of rats with tamoxifen itself or classical inducers such as phenobarbital or dexamethasone stimulated the rate of *N*-demethylation whereas 4-hydroxylation was depressed. We have concluded that by stimulating its own metabolism tamoxifen may accelerate the rate of its own disposal and could also increase the production of a reactive genotoxic metabolite(s). Human and rat liver microsomal preparations are able to activate [^{14}C]tamoxifen in the presence of NADPH to bind irreversibly to microsomal proteins. Protein binding has to be used as an index of metabolic activation and as a surrogate for DNA binding. The extent of binding to DNA appears to be of the order of 50-fold lower than to protein and at the limit of detection using conventional liquid scintillation methods for radioactive detection. Using a panel of 12 human microsomal preparations that had been characterised for the cytochrome P450 content with respect to 9 CYP isoenzymic forms by Western blotting it was shown that CYP3A4 and CYP2B6 were involved with the metabolic activation of tamoxifen to metabolites which covalently bound to protein [11]. This study suggested that the same isoenzymic forms involved in the *N*-demethylation of tamoxifen were also involved in covalent binding whereas 4-hydroxylation reaction was catalysed by CYP2C9. Consistent with the involvement of CYP3A4 and CYP2B6, pretreatment of rats with dexamethasone, phenobarbital or tamoxifen itself caused a significant increase in the rates of protein binding. Although at this stage we cannot formally distinguish if the active metabolites involved in protein and DNA binding are the same, preliminary studies suggest that this is the case.

Comparison between species of the binding of tamoxifen to protein in vitro shows rats to be 3.8-fold and mice 17-fold higher than human liver microsomes [11]. In this respect, the greater

activity in the mouse microsomal preparations reflects the higher levels of overall metabolism. Although the liver was the main site of activation, binding in microsomal preparations from normal human breast tissue could also be detected, although rates were some 7-fold lower than in human liver.

4.2. Clastogenicity in Crespi cell lines

Using a human lymphoblastoma-derived MCL-5 cell line which the human isoenzymes CYP1A1, 1A2, 2A6, 3A4 and 2E1 are functionally expressed, tamoxifen gave a positive result in a micronucleus assay [7]. Using similar cell lines which express individual cytochrome P450s suggested that the isoenzymes CYP2E1 and CYP3A4 were capable of metabolising tamoxifen to genotoxic intermediates as judged by a positive micronucleus test. The Crespi cells expressing CYP1A1, CYP1A2, CYP2D6, CYP2A6 or CYP2B6 did not give positive results over the range of concentrations used with the former 2 isoenzymes [12]. It should be noted that because it has not been established the extent to which the various isoenzymes are expressed within the cells, it is not possible to be categoric that negative results reflect the response in either rodent or human tissues. These results do show that the human P450s have the ability to activate tamoxifen and at concentrations normally found in the serum of women taking tamoxifen therapeutically (≈300 ng/ml).

4.3. Identity of tamoxifen active metabolite(s); role of epoxides

Studies using liquid chromatography with on line electrospray mass spectrometry have detected the presence of a number of metabolites formed from tamoxifen in microsomal incubation mixtures which correspond formally to the addition of oxygen to the drug. A number of metabolites such as tamoxifen *N*-oxide with known retention times on HPLC can be assigned to the peaks observed. Two additional peaks, believed to represent aromatic 3,4-epoxide and 3′,4′-epoxides of tamoxifen have been described (Fig. 3). Although not sufficiently stable to be isolated in quantities sufficient for chemical analysis, in the presence of acid they are converted to the corresponding dihydrodiols, consistent with an epoxide structure. Formation of these epoxides are detected using rat, mouse and human liver microsomal preparations [13]. Other putative active metabolites have been proposed such as a yet unidentified product formed as a result of activation of 4-hydroxytamoxifen [14] or α-hydroxyethyltamoxifen [15]. Support for α-hydroxylation of the ethyl group as a major pathway of tamoxifen activation comes from the observation of a reduced genotoxicity of [D5-ethyl]-tamoxifen. When α-hydroxyethyltamoxifen was prepared chemically and added to rat hepatocytes in culture, the extent of DNA damage, as assessed by ^{32}P-postlabelling, was in the order of 50-fold higher than with tamoxifen itself [16]. The identity of the active metabolite would help to locate which tissues such as endometrium or GI tract, have the potential to activate tamoxifen.

5. Opportunities

The molecular mechanism of action of tamoxifen in breast cancer appears to be a complex mixture of antagonism of the mitogenic action of oestradiol at the level of the oestrogen receptor and cellular effects which may include: (1) inhibition of protein kinase C and calmodulin-dependent cAMP phosphodiesterase and (2) modulation of growth factors including insulin-like growth factor 1 and transforming growth factor beta [17]. Several new antioestrogenic drugs are being developed. Some are based on the triphenylethylene structure of tamoxifen and, like toremifene and droloxifene, do not give rise to liver tumours in rats in life-time bioassays. Raloxifene has a benzothiaphene structure which has antioestrogenic potential [18], while the substituted steroid, ICI 182 780 is a potent specific pure antioestrogen which may offer advantages in breast cancer treatment compared with partial agonists like tamoxifen. These latter analogues, structurally unrelated to tamoxifen, will not have the potential for metabolic activation to genotoxic intermediates. The potential of these newly developed antioestrogens to bring

about the enzyme inhibition and growth factor modulation described above may be central in optimizing their effects as therapeutic agents for the effective treatment of breast cancer in women.

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Toxicology Letters

A few considerations in the design and analysis of experiments in neurotoxicology

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Abstract

To help minimize errors and confusion in the scientific literature, a few principles can be followed. The original intent and hypothesis of each study should be made clear, and deviations from the initial purpose should be stated. The hypothesis-testing portions of the research need to be clearly differentiated from the hypothesis generating sections. If data were selected and reanalyzed in unintended ways, the analyses should be clearly identified as hypothesis generating, and no conclusions should be drawn from such data. The data should be unassailable. The control group should be adequate. The data analysis should be consistent with the experimental design. Given that the larger the number of *P*-values, the greater the rate of false declarations, the total number of derived *P*-values should be reasonable and should be reported. The publication should unveil all details necessary to understand and replicate the research project, including the data analysis. Alternate etiologies should be seriously considered in light of potential confounding factors. It is important to be critical of the results of a study, especially when data confirm a preconceived hypothesis.

Keywords: Causality; Confounding; Control; Double-blind; Error rate; Experimental doubt; Hypothesis generating/testing

1. Introduction

At the Plenary Lecture of the 1994 Society of Toxicology Annual Meeting, Jon Franklin [1] recounted his assignment to cover the Agent Orange 'affair' when he was a journalist at the *Baltimore Sun*. He was given the luxury to have all the time and money he needed to do the story. He went back to original sources, and was horrified when, in his words, he "couldn't substantiate anything ... anything at all". A scientist, referred to as J. Smith, was so outraged by the sensationalistic coverage of Agent Orange, that he spent a day with Jon Franklin teaching him the fundamental principles of toxicology, and providing him with evidence of the unlikelihood of Agent Orange's alleged effects. Jon Franklin had lost a lot of confidence in his profession. He also distrusted toxicologists when he saw some scientists who were backing up the protesters either refuse to release their data, or generate data that were not credible. Then, one day, he ran back into J. Smith who reluctantly confessed that he was working on a grant to investigate the effects of Agent Orange! When scientists support weak propositions for the sake of ideology or money, science and the public lose.

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2. Problems encountered in the scientific literature

When Renaissance succeeded the Middle Ages, a new institution called science was created. It did not take long for it to have its followers and detractors but even some of its adepts had reservations. Robert Boyle (Ref. [2], p. 43) published a monograph in which he wrote "you will find many of the experiments published by authors ... false and unsuccessful ..., you will meet with several observations and experiments, which though communicated for true by Candid Authors or undistrusted Eye-witnesses, or perhaps recommended to you by your own experience, may upon further tryal disappoint your expectation, either not at all succeeding constantly, or at least varying much from what you expected".

Money corrupts and science fraud is very real. Data falsification has been documented not only in industry [3], but also in prestigious academic institutions [4–6]. However, the integrity of science may be more compromised by scientists who unknowingly misuse some techniques, report their data or cite the literature selectively, use the wrong statistical analysis, misinterpret their results, draw unjustified conclusions, and do not distinguish between hypothesis testing and generating.

Another practice that biases the information available on any particular scientific topic is the fact that, in general, negative results are not published. This file-drawer problem, as it has been called [7], is often a consequence of a usually implicit editorial policy under which the majority of published articles must satisfy some arbitrary level of statistical significance. Sterling [8] sampled a couple of psychology journals and reported that 97.3% of the published articles rejected the null hypothesis.

Such a policy adopted by scientific journals not only is antiscientific per se, but also contributes to the confusion encountered in the literature. Grantsmanship does not escape the criticism, it fosters it; the publish-or-perish mandate coerces scientists to satisfy quantity over quality. It also favors generation of new hypotheses to the detriment of their testing.

3. A few fundamental principles in the design and analysis of experiments

Some of the problems alluded to can be alleviated by following some fundamental principles that have often been violated.

1. The original intent of each project should be made clear, and deviations from this initial purpose should be stated to avoid misleading the reader as to the nature of the study. Muller et al. [9] define a hypothesis-testing study as a study designed to confirm or negate a hypothesis. In such a study, the acceptable error rate (α), as well as hypotheses, statistical analyses and eventual transformations should all be specified a priori before the scientist examines the data. Any exception to the previous list makes the study exploratory, i.e. hypothesis generating.

Hypothesis-testing portions of the study need to be clearly differentiated from the hypothesis-generating sections. A hypothesis should not be retrofitted and reported as an original question. If data were to be selected and reanalyzed in unintended ways, the analyses should be clearly identified as hypothesis-generating, and no conclusions should be drawn from such data. Hypotheses cannot be verified with the data that generated them. A conclusion can only be reached after testing these hypotheses with new data.

2. The data should be unassailable. The authors should be qualified enough to understand thoroughly the nature of the collected data as well as the techniques and methods used to generate and analyze them.

3. The control group should be appropriate, i.e. it should be treated exactly in the same manner as the experimental group, except for the independent variable(s) of interest. Studies without a control group [10] cannot be used to draw conclusions. A cross-sectional study where informed consent forms are given to the subjects presents a special challenge because these forms carry a different type of information to the

control and to the experimental groups, and can have effects by themselves [11]; most likely, the control group is told that its role is to serve as a reference in a study, while the experimental group is advised that it is being studied because of suspicion of effects on the variables to be tested. Anomalies in toxicology study control groups have also been documented by Weil and Carpenter [12] in animals. The authors caution the reader against always interpreting statistically significant differences between control and experimental groups as caused by the test compound rather than by abnormal control values.

In a human cross-sectional study, the results of the experimental group can also differ from those of the control group simply due to sampling bias; for example, differences in personality can reflect pre-existing differences between groups, such as those linked to job selection, i.e. people with different personalities and psychomotor skills are not attracted equally to all jobs.

4. The experimental design should determine the type of analysis to be done. For example, if data are collected over time in the same animals, the data should be analyzed with a repeated-measure type of analysis. Analyzing the data separately by time periods is akin to treating the different time episodes as different experiments. Separate analyses would be appropriate if different animals were to be used on the different time periods.

5. More is less. Some researchers design studies with a very large number of questions, and expect to end up with a very large number of answers. What usually happens is that they conclude their study with a very large number of hypotheses, and no answers.

Errors cost, whether they result from false positives or false negatives. Given that the larger the number of P-values, the larger the rate of false declarations of an effect, the total number of derived P-values should be reasonable and should be reported whether these were or were not statistically significant. The actual P-values should be given rather than reference be made to arbitrary levels (e.g. $P < 0.05$). If the author chooses to control α at 0.05 per comparison, for example, and elects not to correct it for the multiplicity of analyses, the author should at least seriously discuss the implications of the adopted strategy in terms of overall error rate. When negative results are reported, power analysis (or other meaningful evidence) needs to show that the methods used can detect an effect of an acceptable magnitude.

6. The authors should provide the reader with enough information about the design, conduct and analysis of a study so that he/she can replicate it. One reason often given for a lack of replication of findings between two studies is the fact that a number of potential differences may account for the observed discrepancies.

7. The effects of confounding variables on the data should be minimized, and their potential impact be discussed. Besides gender, age, education that are usually taken into consideration in investigations involving humans, a number of other factors may affect the outcome of a study, such as, for example, dissatisfaction with the work environment [13], home environment [14], experimenter effect and expectancy [15], placebo effect [16], malingering [17], etc.

Most revealing is the article by Benignus [18] who reviewed the literature on the behavioral effects of carbon monoxide exposure in humans, and found that 75% of single-blind studies were statistically significant, while only 26% of the double-blind studies were. A caution is required, however, as differences in outcome in double-blind studies may still act as an unblinding factor and affect the validity of the results [19].

8. 'Post hoc, ergo propter hoc' is a common fallacy that consists in concluding to a causal relationship in the presence of a simple temporal ordering of events. Another common fallacy is to take a correlation between two variables and to conclude to a causal relationship.

Hill [20] developed a series of criteria used in epidemiology that can be applied to causal thinking in neurotoxicology. These criteria are:

- strength of association;
- consistency;
- specificity;
- temporality;

- biological gradient (dose-response curve);
- plausibility;
- coherence;
- analogy.

Even though none of these criteria can bring indisputable evidence for or against a causal relationship, they help make a decision about its likelihood.

4. Conclusions

The human brain has the remarkable ability to extract coherence out of the randomness of chaos. It will see patterns in the absence of any regularity. It will assume things that do not exist. Caution is therefore de rigueur.

It is Claude Bernard (Ref. [21], p. 73) who reminds us that the fundamental principle of the heuristic method is the doubt. However, Claude Bernard goes beyond this statement and declares that it is necessary to try to disprove a hypothesis once it has been accepted (p. 101). It is important to doubt the results of a study, especially when data confirm a preconceived idea.

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The use of neurobehavioural test batteries for research, diagnosis and screening: methodological aspects

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Abstract

In recent years neurobehavioural tests have been used increasingly in occupational and environmental settings to identify changes in cognitive functioning associated with exposure to neurotoxicants. Applications in cross-sectional research studies, involving the comparison of the performance of exposed and control groups, are relatively well established. However, the use of such methods requires attention to a variety of methodological issues including aspects of study design such as sample size, the selection of appropriate controls, the separation of acute and chronic effects and control or adjustment for numerous potential influences on performance. Studies requiring repeated testing, such as longitudinal investigations or studies of acute effects require attention to learning and practice effects and diurnal rhythms. The validity of adapting existing test batteries for use as diagnostic or screening instruments is questionable. Well-developed techniques exist for diagnosis but this requires lengthy and skilled test administration and interpretation and cannot be accomplished using research batteries. Existing tests may, in the future, be used for screening, but a number of problems will need to be addressed before they can be successfully applied in this context.

Keywords: Neurobehavioural tests; Research; Diagnosis; Screening

1. Introduction

During the last 25 years there has been an increasing recognition that exposure to neurotoxic chemicals may result in subtle effects on the human nervous system which may not be observable in terms of clinical signs and symptoms. The identification of these effects has resulted from the growth of sensitive neurobehavioural methods to assess small alterations in cognitive functioning, which are regarded as behavioural indicators of central nervous system changes. These methods assess a range of cognitive abilities, from relatively simple sensory and motor functions to higher level information processing abilities such as learning, memory and logical reasoning. For most purposes, therefore, a number of tests are put together to form a test battery, which covers different aspects of functioning, although the nature of cognitive abilities is such that inevitably there is some overlap in the functions covered by the different tests.

2. Test batteries

A number of test batteries were originally developed by particular groups to service their own programme of research or diagnostic needs, although several of these have subsequently had wider application. The majority were developed

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in Scandinavia reflecting the origins of neurobehavioural work, which was largely in these countries during the 1970s. They include the original battery used at the Finnish Institute of Occupational Health [1], 2 batteries developed in Sweden, the Swedish Performance Evaluation System (SPES) [2], and the TUFF battery [3], and one developed in Denmark, the Cognitive Function Scanner [4].

Other batteries were developed primarily with the aim of international distribution and application. The most widely used of these are the Neurobehavioural Evaluation System (NES) developed in the United States [5], and the Neurobehavioural Core Test Battery (NCTB) [6], developed in Italy. The development of the latter has been supported by the WHO and it is frequently therefore referred to as the WHO battery. Most batteries now in common usage for research purposes are computer-administered. This enhances standardisation of administration and scoring and reduces the need for a high level of input from skilled psychologists, although this is still required for appropriate interpretation of the results. The NCTB was originally administered in 'pencil and paper' form but is now produced in an automated version under the title of the Milan Automated Testing System (MANS). Both the NES and the MANS have had wide international application in a research context. Both contain the facility for translation into a number of different languages and are supported by a network of information and training.

The majority of batteries in current use drew on existing neuropsychological tests for their development, often adapting these for automated administration. As a result there is considerable overlap in the range of tests employed, although details of presentation, response and scoring may vary. Most batteries will include, for example, a test of simple or choice reaction time, a test of short-term memory and longer-term learning, and a measure of more complex reasoning ability. Outcomes are usually measured in terms of speed or accuracy of response.

By far the most common application of neurobehavioural methods has been in the area of research and for this reason existing test batteries have been developed with the needs of research very much in mind. Alongside research there has also been a desire to use such methods in a diagnostic setting. However, since the requirements of research and diagnosis are somewhat different, this has often led to problems of misapplication and misinterpretation. Most recently there has developed a demand for the application of neurobehavioural techniques in screening and on-going health surveillance programmes. This raises further questions about the suitability of employing existing research-based methods for a variety of other purposes. It is important to be clear about the requirements of each situation in order to ensure that appropriate methodology and techniques are applied in each case.

3. Research

Neurobehavioural methods have been applied to assess both acute and chronic affects of neurotoxic exposure. The former have usually been investigated in experimental settings where individuals are exposed to controlled doses of a particular chemical and within-subject performance comparisons are made to assess the level at which effects begin to occur [7]. This type of work allows the control of a range of factors with the potential to influence test performance.

By contrast, the majority of investigations of chronic nervous system effects have involved cross-sectional field studies where the test performance of a group of individuals who are regularly exposed to a particular chemical (occupationally or environmentally) is compared to the performance of a non-exposed control group [8–10]. Since cognitive performance is represented by scores on a continuum (for example response speed in ms) an effect of exposure is assessed in terms of the size of the difference between the scores of the 2 groups. In particular, confidence in the results is strengthened if a dose-effect relationship can be demonstrated.

It should be noted that, in this context, no attempt is made to define 'normal performance' or to analyse results in terms of the number of

'abnormal' individuals in each group. Scores on tests of cognitive function are heavily dependent on cultural, educational and pre-existing intellectual factors and universally applicable reference criteria are thus impossible to determine. In studies of this type it is usual to exclude individuals whose history suggests the presence of effects on the nervous system unrelated to exposure, for example those suffering from diagnosed nervous system disease, or those with a history of serious head injury or substance abuse. In particular, however, the suitability of the control group is of paramount importance. It is normal to ensure matching in terms of age distribution, gender, educational level and social and cultural background, as well as in terms of the type of work usually undertaken. Other potentially important factors such as the influence of lifetime alcohol consumption and smoking history may require subsequent statistical adjustment. Assessment of the influence of intelligence level prior to exposure is particularly problematical. In the past this has usually been carried out using a verbal test such as reading ability or vocabulary, on the basis of evidence that scores on such tests correlate highly with IQ, as conventionally measured, and are relatively resistant to nervous system insult [11]. More recently, however, this view has been questioned and investigators are increasingly favouring educational level as a more valid indicator of this factor.

In addition to the characteristics of the subjects certain situational factors also require careful consideration. Some of these relate to the time at which testing is carried out, specifically to control for the effects of circadian rhythms on performance [12] and the potential confounding of acute and chronic effects when testing is carried out very shortly following exposure. In the case of many substances data on the persistence of effects following exposure do not exist and, where chronic effects are under investigation, pragmatic decisions may have to be taken as to when acute effects may safely be assumed to have disappeared.

Other situational factors have a direct bearing on the particular tests employed. In a research context major emphasis is placed on the standardisation of test administration and on unambiguous, quantitative scoring methods in order to acquire reliable data. In addition, tests must be economical in terms of time amd personnel, relatively quick to administer, inexpensive and portable. Further they must be acceptable to ostensibly healthy individuals who do not perceive themselves to be in a patient role. All these factors tend to limit the choice of tests.

In practice the ideal research battery is fairly short, is easy for non-specialists to administer and score and has high reliability and sensitivity. While existing batteries tend to fulfil these criteria, however, they are perhaps inevitably limited in their capacity to define the specific effects of a particular chemical in precise psychological terms. While, therefore, many of the earlier methodological difficulties of research in this area have now been overcome the challenge for the future is to incorporate a more 'diagnostic' (at group level) element into research batteries by including tests which are more theoretically interpretable. This is likely to be achieved by the increased application of techniques developed within the framework of cognitive research into normal intellectual functioning. Such techniques allow the analysis of patterns of performance and the identification of subtle shifts in these patterns, an approach which may be particularly suited to the needs of neurobehavioural research. Examples of test batteries which place more emphasis on this type of approach are the Information Processing and Performance Test Battery [13] and the Automated Cognitive Test Battery [14]. Such an approach necessarily implies a move away from some established neuropsychological tests which in the past have been adapted for use in a research context, and are retained in many existing research batteries, but which may be better suited to a diagnostic setting.

4. Diagnosis

As noted above many of the tests included in neurobehavioural batteries are adaptations of neuropsychological tests which have been used

for many years in clinical diagnostic settings. For example, several tests have been borrowed from the well-established Halstead-Reitan Neuropsychological Battery [15]. However, in considering the question of individual diagnosis it is important to note that the automated forms of these tests are necessarily *adaptations* for research purposes and as such can no longer be considered to be diagnostic instruments. Many would not be suitable for research in their original form, for example in terms of length, non-standard scoring or simply the impossibility of automated administration because of the particular stimuli or response forms required. In their adapted form, however, they go some way towards fulfilling the objective of research which is to identify neurotoxic effects at group level.

The objectives of diagnosis, however, are substantially different. Here the concerns are to establish whether an individual has experienced a deterioration in cognitive functioning and whether that deterioration is associated with neurotoxic exposure. This requires much more lengthy and detailed examination of an individual's level of functioning and reference to their clinical, social and exposure history. The approach is likely to be a multidisciplinary one, involving a range of professionals including hygienists, toxicologists and physicians as well as the neuropsychologist.

Those administering psychological tests in such a setting are able to employ them successfully for diagnosis because they place qualitative information and clinical judgement alongside the quantitative information which the tests provide. Limited automated testing, by contrast, as provided by research batteries simply produces numerical scores which are unlikely, alone, to provide sufficient information on which to make a clinical decision.

While many of the factors which are taken into account at group level in a research study, for example alcohol consumption and smoking history, will also be considered in a diagnostic setting, the diagnostic approach is essentially one that involves 'case' definition and as such requires specific assessment techniques which should not be confused with those employed in a research context.

5. Screening

Recently there have been suggestions that existing widely used test batteries might be usefully employed as initial screening devices to identify individuals requiring further investigation, or to follow up those identified as being 'at risk' because of potentially hazardous exposure. Although at first sight this seems an appealing prospect the above discussion of the nature of neurobehavioural testing immediately highlights a number of difficulties inherent in this approach.

The practical demands of screening large numbers of individuals indicates that those tests used in research batteries are most likely to be appropriate for this purpose. However, as noted above, the outcomes of these tests are typically represented by scores on a continuum and the distribution of scores for any population will depend on a number of factors noted earlier such as age, educational level, etc. Despite initial optimism about the development of 'normative data' associated with the most widely used batteries, it has become clear that this is not a realistic proposition. As a result it is not possible to define a cut-off point for use as an indicator of impairment which would be applicable across widely differing populations and which would presumably be a requirement of a screening programme.

Conversely those tests which have the potential to identify a 'case' are lengthy and complicated, requiring a high level of input from a skilled neuropsychologist. This is clearly incompatible with the demands of a screening programme. Those screening instruments which have been successfully employed in the workplace to date, and which come under the broader heading of neurobehavioural methods, are questionnaire-based, for example the Orebro Q-16 [16] designed for the detection of solvent-related neurotoxicity. The development of a screening technique based on cognitive testing continues to represent a considerable challenge for the future.

6. Summary

In summary, the appropriate use of neurobehavioural measures requires attention to the

objectives of the exercise since different techniques are required in different settings. Diagnostic methods are well established but, when properly applied, are inpractical in a research context. Similarly the attribution of diagnostic properties to research techniques is inappropriate. Future developments in research methods should allow more precise definition of the effects associated with specific exposures at group level, however, and this information is likely to be of value to those engaged in individual diagnosis. Most difficulties in the application of neurobehavioural methods lie in the areas of screening and health surveillance which appear to demand a combination of the features of both research and diagnostic approaches. The reconciliation of these demands represents a considerable challenge to those involved in the future development of this area.

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Neurobehavioral assessment in toxic injury evaluations

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Abstract

Forensic neurobehavioral evaluations present special validity problems such that standard assumptions, procedures, histories, and fact-finding methods used in treatment settings may be inappropriate and misleading. This article discusses basic issues in assessing the quality and reasonableness of forensic toxic injury evaluations. Topics include selection of test instruments, history, functional assessment, threats to validity, voluntary manipulation of test results, chemophobia, limitations of the state of the art in neuropsychology and neurobehavioral assessment, base rates, and norms. Understanding the roles these topics play in toxic injury evaluations is essential to making informed judgments about the quality of forensic neurobehavioral evaluations.

Keywords: Neurobehavioral assessment; Neurotoxic; Disability; Neuropsychology; Forensic; Solvents; Litigation

1. Introduction

Application of neurobehavioral procedures in forensic contexts (personal injury claims and litigation) poses special challenges to scientifically oriented clinicians, and requires awareness of concepts outside the range of normal clinical and scientific evaluation. An understanding of these concepts is critical to judging whether findings from forensic neurobehavioral examinations are reasonable. Knowing the purpose of neurobehavioral testing in toxic injury evaluations is the first step in this endeavor. The purpose of neurobehavioral assessment in toxic injury cases is to evaluate the patient's current status and to describe the differences between the patient's neurobehavioral functioning pre- and post-exposure.

There are certain essential questions which are addressed in most neurobehavioral assessments of toxic injury. These include whether there is a neurotoxic injury and, if so, its nature and extent; the cause of injury; the impact of the injury on important cognitive functions, sensory and psychomotor functions, and personality; whether the injury is treatable and, if so, through what procedures; the prognosis; and other consequences of the injury.

2. Patient history

Thorough, accurate histories are necessary if we are to understand the causes and effects of neurotoxic injuries. Reliance on inaccurate or incomplete histories produces misleading and erroneous conclusions. An extensive array of pre-injury health variables confronts the examiner in forensic neurotoxic cases.

Studies of neurotoxic injuries are further com-

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plicated by political, legal, and media forces associated with neurotoxic litigation [1–9]. Lawyers have conferences with patients prior to examinations, and decline to disclose the nature of their influence on the grounds that such discussions are privileged. Some attorneys openly admit that they coach clients specifically in preparation for psychological testing [10,11].

Problems with history which invalidate our opinions include inaccurate estimates of pre-injury functioning caused by reliance on unreliable sources, missing information about pre-existing impairments and prior injuries, inaccurate assumptions regarding pre-injury cognitive capacities, lack of awareness of psychosocial stressors influencing the patient's performance, incomplete data concerning psychological problems or mental disorders, and the widespread tendency for clinicians to presume etiology based on lay patients' conclusions about etiology [12–15].

3. Confounding factors

Numerous confounding factors complicate forensic neurobehavioral assessments, in addition to the usual challenging confounds faced by investigators in this field (e.g., see [16–26]). Among those prominent in recent cases are influences of pre-existing problems and psychological deficits, use of inappropriate norms, chemophobia, response biases, third party influences, and occasionally voluntary data manipulation.

3.1. Concurrent confounders and pre-existing problems

Problems interfering with valid assessments include concurrent confounders and pre-existing problems. Pre-existing problems such as learning disabilities or prior injuries, when unknown to the examiner, may lead to conclusions that impairment resulted from a toxic exposure [25,27]. Distraction and pain may confound test scores. Thought disorders are associated with dramatic disruptions of neurobehavioral functioning. Numerous neurotoxic patients utilize psychoactive substances which affect neurobehavioral test data. Medications such as antidepressants, anti-anxiety agents, analgesics, sedatives and soporifics all may affect scores on neurobehavioral tests. Pain and emotional problems are a frequent source of contamination of these data. Anxiety and mood disorders in particular are common confounders in forensic neurobehavioral assessment [28–30].

Neurobehavioral assessment presumes the best possible performance on the part of the subject [31]. Motivation has a profound influence on test results but is a relatively neglected area in the literature. For example, voluntary manipulation of neurobehavioral tests is simple for most untrained examinees [32–35]. In forensic cases, the standard of practice is to consider the possibility of malingering [36]. However, most neurobehavioral tests lack validity scales or internal measures for evaluating the level of cooperation of the patient. External incentives and social influences need to be carefully considered by forensic neurobehavioral examiners, and a great deal more research is needed to address these problems.

3.2. Use of unreliable norms

Use of inappropriate norms is a frequent threat to validity [37–39]. In many respects, we simply do not know the appropriate normative standard. As Reitan (1992) observed, neuropsychologists simply do not know the normal rates of neuropsychological phenomena, and as a result, the boundaries between normal and abnormal behavior are unclear [40]. It is often unclear what percentage of cases are clinical injuries, normal variations, or byproducts of various confounds. Recently improved norms have been introduced which are more representative of the general population [41] but their use remains controversial, e.g., the issue of how well they apply to brain-damaged patients [42].

Base rates of neurobehavioral symptoms have received little attention in the neurobehavioral literature, although recently normative data have begun to appear (e.g., see [43–45]). Without awareness of prior probabilities of symptoms, interpretation of presenting complaints is ambiguous and opinions as to causation of pathol-

ogy are confounded [46]. Unequivocal neurological abnormalities are common in asymptomatic patients, and in the general population neurological problems are a frequent occurrence [47]. Statistical variation is often confused with injury.

Because of the ambiguity of underlying base rates, the significance of self-reported symptoms is not clear and should not be relied upon as confirmatory evidence of injury to the brain [42]. Similarly, due to our limited knowledge of base rates, test sensitivity and specificity are not clear. The positive predictive power of a test is often estimated by clinical judgment with no explicit empirical foundation [46]. There are extensive unmet needs for epidemiological data pertaining to neurotoxic phenomena, and for research clarifying the influences of confounding factors affecting neurotoxic allegations (e.g., see [48,49]).

3.3. Problems associated with estimating pre-injury functioning retrospectively

Pre-injury neurobehavioral functioning is commonly inferred from occupation, but inferences about individuals in many forensic cases tend to be more speculative than informed. Many testifying experts are not familiar with the cognitive and intellectual requirements associated with specific occupations, and do not follow the extensive literature describing such requirements (e.g., see [50]).

Occupation may be a greater confounding factor than has been recognized in the literature. Most of the neurotoxic literature is based on studies of groups which are average to below average on most relevant cognitive variables, in comparison to the normal population, and thus the cases studied should not be expected to function in parallel to the overall population distribution even in the absence of toxic injury. An extensive literature is available for researchers to identify expected levels of proficiency in functions important in neurotoxic assessments. For example, psychomotor functions, strength, intelligence, numerical and verbal capacities, and many other cognitive and physical capacities have been studied for many thousands of occupations [51].

This literature offers a valuable resource for experts needing to estimate pre-injury functioning, for researchers wishing to control for occupational confounders, and for treating clinicians and rehabilitation personnel attempting to determine whether the patient will be able to work in certain occupations in the future.

Experimental subjects and controls are drawn from different occupations which vary substantially in capacities directly relevant to the tasks performed in neurobehavioral assessments [50]. For example, some neurobehavioral tests require use of one's fingers primarily (as distinct from primarily the hands or entire upper extremities), e.g., the Santa Ana and Perdue Pegboard. Strength and fatigue are a part of other tests, such as grip strength testing. Virtually all are correlated with general intelligence to varying degrees [37,38]. Many are affected by clerical aptitude, e.g., symbol digit. Yet there is very little neurobehavioral test data with which to rule out indirect practice effects and other differences in ability which are associated with occupation.

Printers, painters, electronics workers, degreasers, dry cleaners, field crop farm workers, and general farm workers' jobs require average to low average intelligence and motor coordination [50]. These occupations vary from frequently requiring fingering of materials and objects to no significant fingering activities at all. They predominantly require verbal and numerical aptitudes in the low average range. Their clerical demands range from average to the lowest 10% of the working population. Strength demands vary widely, from rarely to frequently requiring lifting, handling and gripping heavy objects. Not one of these occupations requires above average intelligence, clerical aptitude, motor coordination, or finger dexterity [50]. Convenience samples employed as controls may work in the same general area or for the same company, but they may not have identical cognitive and physical capacities.

4. Limitations of the state of the art

There is limited research on cognitive-social factors influencing neurobehavioral assessment. Yet there is ample reason to suspect that cog-

nitive-social variables are at play to a substantial extent in toxic injury assessments [1,3–8,52–55].

Another difficulty with drawing conclusions about individual injuries is that the literature deals with statistically significant conclusions about groups for whom the absolute mean differences between the groups are often small and the overlap between exposed and unexposed groups substantial. Attempting to make point and bandwidth predictions about individuals based on general findings of subtle differences between overlapping groups is more an art of speculation than scientific logic.

There is no generally accepted definition for impairment, and conflicting definitions are employed in forensic cases. Test authors do not all use the same standards, even for ostensibly identical or similar functions such as intelligence and memory. There is a dearth of empirical data supporting the use of particular cutoffs for concluding that deficits exist, with the current trend being to rely on relative standing in the population rather than the relationships between test data and real world functioning [41].

Tests drawn from the neuropsychological literature were typically validated with comparisons between normals and obviously grossly impaired samples. But forensic cases most commonly involve patients with more subtle impairments, so expert conclusions offered in the courtroom often do not follow from the literature.

A troubling problem in toxic injury evaluations is the tendency of some psychologists to refuse to disclose their data for scrutiny by the scientific community at large (e.g., see [56]). Some psychologists argue that only other clinical psychologists or neuropsychologists are qualified to form opinions about their work. Errors which are readily apparent to scientifically trained physiological psychologists, toxicologists, epidemiologists, and other highly qualified neurobehavioral scientists go undetected because of this intellectually embarrassing requirement. Ethical scientists should make efforts to oppose this secretive use of data; science in a closet is not science.

Attorneys routinely ask for opinions about causation of deficits reflected in testing, but the current state of the art of neurobehavioral tests permits measurement of functions without providing direct evidence of the causation of deficits. Causal relationships are deduced from the context. Although testifying experts at times assert that there are signature patterns of test scores associated with specific toxic substances, there exists no encyclopedia or compendium of such patterns established empirically.

5. Litigation factors

Patients in litigation appear to behave differently than similar nonlitigating patient populations in a number of respects. For example, they appear to report more symptoms than controls and score more in an impaired direction on neuropsychological tests. Litigation is associated with higher base rates of neurotoxic symptom reporting and reporting biases in history. Experts who wish to serve as testifying witnesses need to study the base rate literature and the emerging literature on social, cognitive, and contextual influences on plaintiffs [1,43–45,52–55].

6. Conclusion

The field of neurobehavioral assessment of toxic injuries is a fascinating, burgeoning field of investigation. Although subject to numerous validity problems typical of a young discipline, the area is a promising one for future research. In particular there are needs for reliable measures of functions which permit prediction of performance in activities of daily living and employment, emotional distress and life satisfaction, and outcome of treatment procedures. Another important area needing study is the influence of contextual, social, and cognitive factors associated with litigation. Finally, there is a pressing need for symptom validation procedures. The field of forensic neurobehavioral assessment provides extensive opportunities for researchers and other scientifically oriented professionals to make a valuable contribution to society.

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Neurobehavioural test batteries: current status, critical evaluation and new directions

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Abstract

Neurobehavioural techniques are currently making a contribution to knowledge in toxicology which is being used to set safety limits of exposure to hazards. This, however, puts pressure on researchers in the field to ensure that the methods used are the best and most informative available. A review of the neurobehavioural tests currently in use shows that there are a number of issues that need to be addressed to strengthen and increase their utility. Issues include test selection and the validity, reliability and sensitivity of tests, bias and confounding of measurement in the testing session and problems in interpreting the results.

Keywords: Neurobehavioral methods; Behavior

1. Introduction

In the assessment of effects of toxic exposure, neurobehavioural testing is beginning to come of age. Increasingly, studies are using neurobehavioural techniques to look at the effects of exposure to potential toxicants for an increasing range of exposures. Despite the very healthy growth, the area is in danger of slowing and stagnating because of an apparent tendency to avoid facing some critical issues mainly relating to problems of confounding of test measurement. Now that the field is starting to mature, it is imperative that we tackle some of the issues in a systematic fashion. This paper focuses on the reasons why we may not be getting accurate measures of behaviour change in the face of toxic exposure and why our interpretations of behaviour change may sometimes be misleading.

2. Problems of making measures of behaviour

2.1. Informed consent

Increasingly, due to ethical considerations, neurobehavioural toxicity testing requires informed consent from study participants. This can bias behaviour measurement through selecting out only volunteers. To avoid such bias, where possible testing should be mandatory even though this often contravenes other ethical practices. Alternatively, we need to establish that non-participants do not differ from participants on any important variables. For example, only workers who have relatively low exposure to a hazard may volunteer, so biasing assessments of the neurobehavioural function of the group.

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2.2. Effects of motivation to perform

Motivational effects can take 2 opposite forms, subjects trying to do very well (faking good) and trying to do poorly (faking bad). Faking good may be less of a problem in testing for toxic exposure effects where the potential for compensation payments is a possible reason for changing motivational state. Even so, in many situations some workers will try harder than others, with consequent effects on test performance. It must be remembered, however, that all behaviour requires a certain level of motivation or incentive to generate it. This is particularly so for tests involving memory or attention.

Research on motivation suggests, however, that raising motivational level may not actually produce large changes in performance. For example, monetary incentives improved information processing performance to a greater extent than did practice on the tests but this effect was only seen for a composite measure of performance [1]. Incentives did not change the relationship between performance on information processing tests and intelligence tests, indicating that manipulating the emotional state of the individual did not result in changes in mental capacity. For some tests, trying harder will not improve performance much and can even make performance worse. Trying harder on a relatively easy task is simply not possible [2]. The effects of trying hard can sometimes be seen, however, in tasks in which a speed-accuracy trade-off can occur [3].

The opposite end of the motivation spectrum, faking bad or malingering, is often raised as a serious difficulty for the validity of neurobehavioural testing, because there is often considerable payoff for occupationally or environmentally exposed individuals to make the most of, or exaggerate, any effects of a neurotoxicant. In an investigation of the relationship between monetary incentives and poor performance [4], subjects were given varying levels of information about how to fake poor performance on a forced-choice memory test and studied test performance under circumstances where motivation to fake was manipulated by monetary reward if their faking could not be detected. Faking subjects produced poor performance consistent with the nature of instructions on how to fake but monetary reward by contrast did not produce any additional negative effects on performance.

A few techniques have been used to assess the possible confounding effects of motivation level. The overall approach is conservative in attempting to detect individual subjects who may be judged as 'faking'. Techniques include methods such as forced-choice tests [5], examining the pattern of test performance such as variations in the speed-accuracy trade-off [3] and using discriminant function analysis [6]. A method which has not been used in the assessment of neurotoxic action involves Signal detection methodology [7]. This technique differentiates sensitivity to detect the signal from the subject's bias or criterion which is their expectation about the likelihood of the correct response occurring. Measures of sensitivity are likely to remain constant over situations where a subject's criterion setting might be affected by such factors as external motivation. Consequently the method would be extremely useful for both detecting the existence and the extent of the motivation effects.

2.3. Classical confounders

2.3.1. Test situation

A large number of influences in the test situation have been shown to affect performance. All relate fundamentally to the general state of the individual. Where the factor makes significant changes to the individual's level of arousal, resulting in either too little or too much, performance would be expected to suffer [8]. Evidence is available that factors such as how hot or cold it is, the effects of background noise, the lighting level and the time of day can effect performance [8]. While most studies attempt to control for these factors by holding them constant or by counterbalancing, test situations such as those based in industry settings may make such actions difficult or impossible. Clearly variations in the test situation must not be overlooked

as they can have relatively large effects on performance.

2.3.2. Testing procedures

Factors due to the test procedure may also be important. For example, the importance of procedural rigour was demonstrated in a study of the covariates of peripheral nerve function using vibrotactile and thermal thresholds and nerve conduction velocity and amplitude [9,10]. For all measures, the examiner emerged as a major covariate, with the difference between examiners being as much as one standard deviation in magnitude for some measures. It is not clear why the examiner exerted such a large influence on these measures as they differed in the degree of subjectivity they involve, however these studies strongly suggest that measures should be as objective as possible. Methods such as computerised testing, standardised protocols and interrater reliability need to be brought into play here to avoid these sorts of problem.

2.3.3. Effects of repeated exposure to the test

It is well known that repeated exposure to a test can influence performance through either the effects of practice or fatigue. For many tests the effects of practice or of fatigue are simply not known. Test performance can be changed significantly due to both practice and fatigue. Evidence shows that a single repetition of a test can produce a practice effect which is sufficient to counter the effects of 20 or more years of ageing [11]. For all tests, asymptotic levels of performance are reached before measures are taken to represent stable performance [12], and taking care to avoid long periods of testing. However the time taken to benefit from practice and to show fatigue-related deterioration in performance can be used as a useful measure of toxic effect. The signal detection method described above would also be useful for establishing the effects of fatigue on performance.

2.3.4. Characteristics of individuals

Differences between individuals are most often raised as potential confounding factors. These include the person's age, their level of education, and the extent to which they use alcohol. While most studies attempt to control for these factors, they often do so without establishing either the extent of the influence on test performance or that the factors actually exert unwanted influences on the tests in question over the age-range of the study group. Not all functions nor all aspects of functions are affected by age, unless the age differences are very great. For example, it appears that motor performance slows and becomes more variable with age [13], but memory capacity may not [14]. Factors like practice and physical training have also been shown to minimise the age-related differences between old and young adults [13]. Studies showing differences in motor performance due to the age-related decline compare very great differences in age, with the older group typically in the 60 plus age group. Most studies of neurobehavioural effects involve subjects who are considerably younger, particularly if focusing on exposures at the workplace. Consequently, age-related differences in function may not be a significant problem in many studies.

These issues are also relevant to other assumed confounding or nuisance variables. Taking action to remove the effects of these variables may not simply be a waste of time, particularly if post-hoc statistical methods are used. If the assumed confounding variable is significantly correlated with exposure to a neurotoxicant, as is often the case with factors like age, education level, and alcohol use, attempts to reduce the effect of the variable can also reduce the power to detect a difference between exposed and non-exposed subjects [15]. It is important to analyse the relationships between confounding variables and both dependent and independent variables before designing a study.

3. Problems of making particular types of behavioural measures

Particular types of measures may have particular problems or confounders associated with them. Standard neuropsychological tests are often used in studies because there is information

on their validity and reliability [16,17]. The detection of neurotoxic behavioural effects, however, places a number of constraints on the use of both standard and novel tests.

3.1. Expectations of a test

In many assessment situations the perceived aim or face validity of a test may have an implicit influence on test performance for some individuals. For example, the motivation to perform the test may depend on the subject's expectations from his/her experience of the test. Factors like the title of the test and the level of complexity of the test (perceived as too easy or too difficult), may cause subjects to change their performance in response to their expectations about any consequences of particular performance on a test. The extent that these features influence performance is not understood. Clearly situational factors may also play a role, but at the present time we do not know how big a role. There is a need for some careful research on the influence of these implicit features on performance including the face validity of tests.

3.2. Test representation and uniqueness

Two essential features of good measurement are the extent to which the measures generated are reliable and valid. Good test reliability is particularly important because errors of measurement can obscure the relationship between dependent (test performance) and independent (exposure) variables. Test developers and users in this area have used tests regardless of the reliability of tests and measures. For example, one study [18] demonstrated that test-retest reliabilities were only moderate for a large number of measures from the Neurobehavioral Evaluation System (NES) [19], one of the most commonly used test batteries. This has implications for the utility of this test system in many settings. In contrast, the approach used in the development of the automated performance test system (APTS) [12] selected tests on the basis of their stability over practice for means, variances and intertrial correlations. The measures used in these batteries have the strength of having known, high reliabilities available before the measures are used to any great extent. Kantowitz [20] makes the point, however, that this approach does not deal with the issue of validity or uniqueness of measures. He questions the common practice of classifying measures into perceptual, motor or cognitive domains, in absence of any theory to guide test or measure selection. He argues that functional groupings are somewhat arbitrary, with little other than expert judgement to support them, and they often overlook the range of functions required to perform each test and consquently hamper interpretation of test results. For example, most tests in neurobehavioural toxicology rely on visual stimulation, a fact that is usually overlooked in interpreting study results. If the toxicant does have effects on the visual system, these could be fallaciously interpreted as deficits in another functional domain such as motor performance or memory. It has been demonstrated that visual impairments can indeed adversely affect performance on tests which use vision as a vehicle to deliver test stimuli to other parts of the cortex [21].

A number of authors have put forward an alternative approach [15,20,22,23] maintaining that the field needs tests that relate to an overall model or theoretical framework. Often the choice of tests is defended on the grounds that they are a pragmatic representation of what functions might be affected based on previous research. Kantowitz [20] in contrast maintains that in selecting human performance tests 'theory is the best practical tool'.

3.3. Sensitivity of tests

Tests need to be sufficiently sensitive to detect neurotoxic effects where they exist. Tests with high reliability and good validity will help to reduce the error variance so as to reveal effects due to the independent variable, exposure. Tests from traditional batteries may appear to be useful as they usually have good psychometric

qualities. They are, however, designed to assess clinically affected individuals, and are not challenging enough or do not have a large enough range of measurement to pick up deficits before they become either irreversible or too limiting to the individual's function. Alternative test methods for performance testing should be considered including more complex tests such as dual tasks and grammatical reasoning, self-paced testing [24], adaptive testing [25] and signal detection theory [11].

The issue of sensitivity in this area is rather like walking a tightrope. From the view point of interpretation, there is a limit to increasing sensitivity of tests. Tests can become too sensitive when they detect differences between individuals due to irrelevant factors such that the effects of the independent variable can be overshadowed. The real issue here is the acceptable level of concern. When is the size of the exposure effect large enough to require action? Simply showing that exposed groups are different from non-exposed may not be sufficient in practical terms if the size of the difference is not taken into account. Choosing a criterion for judgement is not easy. Some authors suggested that the effects of aging constitute a good criterion [26,27]. Other alternatives include effects on daily functioning and quality of life [15] and methods to assess delayed neurotoxic effects [28].

In a few instances researchers have tried to anchor the size of performance deficits in terms of the effects of an agent which is known and accepted to be neurotoxic. For example, 2 groups have developed dose-response curves for the effects of alcohol on performance in their test batteries [12,28]. In both cases the aim was to create a form of calibration or criterion validity on which effect sizes of various exposures could be judged. It does, however, still beg the question of when we should be concerned. Should it be at the equivalent of 0.05 blood alcohol, or one night without sleep? The issue of practical consequences of exposure effects which are picked up by sensitive tests is an important one. We need to pay attention to it if neurobehavioural testing is to be truly useful in toxicology.

4. Problems of the interpretation of behaviour

Issues also exist around the interpretation of the results of neurobehavioural tests. These issues mainly stem from problems due to the nature of the study design and the consequent analysis of test results.

4.1. Purpose of the study

Studies of neurobehavioural effects are conducted with 2 main purposes, to screen or establish whether particular individuals are effected by exposure and to diagnose or determine the nature of the effect. It is argued that these 2 purposes have implications for the inclusion of different types of tests in a battery [29]. Tests for screening need to be suitable for detecting the specific effect of the toxicant. Diagnosis, on the other hand, requires a much more comprehensive battery in order to look at the range of possible effects of the substance. The problem is that the diagnostic purpose really needs to be done first and done well in order to establish the best tests for screening individuals. The effectiveness of screening depends to a great extent on the effectiveness of diagnosing what are the real effects of the toxicant. Too often, in this field, the diagnostic work is largely equivocal or does not clarify which specific effects are characteristic of the neurotoxicant. The screening approach is taken too early. As a consequence it leads to poor screening and is probably one of the reasons for the lack of resolution about the effects of particular neurotoxicants such as organic solvents.

4.2. Individual differences in basic capacity

This is an issue because studies in neurobehavioural toxicology tend to be cross-sectional in design. Attempts to match exposed and control groups on confounding variables may not be very successful. A variety of methods have been used to control for baseline intellectual capacity, including pre-existing intelligence or vocational test results taken before any toxic exposure had occurred [30], the use of monozygotic twins [31]

prospective study designs in which baseline non-exposed performances are measured and compared to performances at intervals over a period of exposure years [32] and through use of measures taken currently but judged to represent basic intellectual capacity such as the vocabulary test from the WAIS [33]. The first 2 methods can be difficult to achieve so have not been used very frequently in neurobehavioural toxicology studies. The last method is used much more often, but presents some particular problems as it is very likely that the measures of basic capacity taken after exposure may also be affected by exposure [34]. In fact it is possible that any measure of intellectual capacity may be affected by the neurotoxicant so making it impossible to use these measures to show prior baseline functioning once exposure has already occurred.

5. Conclusions

Neurobehavioural testing is at an important place in its development. It is now clearly established and accepted as a methodology which can add to the body of knowledge about how toxic exposure can affect health and safety. Unfortunately, however, there are many loose ends to this development. Many issues are unresolved and are increasingly being ignored in favour of a pragmatic approach, 'let's get measuring'. Now is the time, however, for us to reassess our tools and to attempt to build up the strengths and work on the weaknesses. Issues associated with the choice of tests, reliability, validity, sensitivity and the role of confounders must be addressed before the field can move on.

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Media and risk commumication

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Keywords: Communications media; Risk reporting; Toxicology; Journalism

The communications media in Western societies play an essential part in risk communication to the public. Several surveys have shown that a majority of people get most of their information in this field from the media. Risk reporting has during the past two to three decades had a strong tradition in the attention of the media, occupying a lot of different kinds of journalists and journalism. The main actors when it comes to chemical risks and toxicology are specialists in environmental journalism and medical reporting, but the issue is also handled by many other media players such as journalist experts in labour-market and labour–environmental problems, in traffic and communication, in politics, in consumer journalism and in scientific writing in general.

However, like the toxicologists, media people are surprisingly unaware that they are in fact communicating risk to the public. We do not talk in those terms; we do not call each other risk journalists; we do not have seminars or special education in risk reporting. One reason is probably lack of knowledge about risk research and the fact that risk stories are only one part of the job even for environmental and medical reporters. Sometimes this lack of risk-thinking becomes very obvious, for instance after the Chernobyl accident, which in Sweden and many other countries created very confused reporting where people were left with a lot of unanswered questions, anxiety and uncertainty.

Nevertheless, risks have been on the media's agenda for a long time and it will continue to be so. As with most other topics we choose to cover and to transform into news, the media mainly reflect trends and developments in society; they very seldom create their own issues. The media seldom have the initiative. We pick up what is happening and we use the public interest as the main impelling force.

When it comes to risks it is really rather simple. Risks are of great interest to the readers, the viewers, and the public. That's why we write. Good health is the most important thing in life for most people, as many surveys have shown. We have a society more and more preoccupied with health, bordering what some people call health-fascism. Health has for some people become a sort of religion and we have a powerful medical profession that give promises to create the tools for this. To give proper information and knowledge on health issues to the public is therefore an important task for the media.

The undeniable fact is that health and en-

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vironmental risks and lifestyle are linked. Doll and Peto in their book *The Causes of Cancer* (1980) stated that about 70% of cancer is due to environmental risks, mainly smoking and nutrition. The same risk factors provide 60% of the explanation for cardiovascular diseases. And those two diseases are undoubtedly the dominant causes of illness and death in Western countries.

In the same way, environmental problems come high up in people's judgement of political important questions. They influence political elections, and no political party can any longer ignore the environmental issue. In almost all European countries Green parties have entered parliament. The environmental movement is strong and the focus is more and more on individual responsibility to solve problems. You have to fulfil environmental demands and behave correctly, recycle, produce less garbage, use less gasoline, drive less and so on. This outlook creates the same need as do health issues for proper knowledge and information.

In the medical field, modern media reporting on risks dates back to the 1960s, when side-effects of drugs became a big issue in the media as a reaction to the dreadful Thalidomide disaster. Both the drug industry and the medical profession were surprisingly unprepared for this discussion, and the conflict impaired the relations between media and the industry for a long time: perhaps it still does. But the demands for openness won. Today it is not news that drugs create side-effects, and people can obviously handle the necessary risk-evaluation.

Pollution and environmental and chemical risks in the workplace were two other big issues in the 1970s, fuelled by the development of Ames tests and other mutation assays. That was the time when many scientists warned about the threat of a cancer epidemic as a result of the new chemical society, and tests revealed more and more compounds to be mutagenic. In the media we reported about cancer risks with one substance after another. We sounded the alarm and cried wolf many times. Probably one explanation for the strong belief among the public that chemical substances and additives are the main cancer risk is the media coverage from those days.

Doll and Peto put the cancer risk area into its proper perspective, and since then the 'alarm reporting' has slowly and gradually matured into a more sophisticated health reporting and a greater critical consciousness in the media. The growing scientific influence from modern epidemiology has also contributed to this change, as we journalists learned to handle the special epidemiological world with its RR-figures, risk factor-thinking and confounding problems.

In the past 10 years the AIDS epidemic has challenged the ethics and responsibility of the media. AIDS reporting is basically a gigantic case of risk reporting.

How should media handle risk communication practically? We can only use the same journalistic traditions and methods as we do in all other reporting. The most important is that articles have to fit in the media concept, mainly the focus on news. The issue has to be presented as news. That is the reason for publishing, and journalistic professionalism decides how to make risk reporting fit these demands.

To guarantee that reporting will be as good as possible, the following factors are vital.

- A capable reporter with experience and knowledge and awareness of the very specific ethical problems in risk reporting. The ethical demands are, for instance, never to forget to distinguish the big risks from the small ones, to have the courage to refrain from writing about indifferent and irrelevant things, and also to try to avoid confusing readers unnecessarily or causing them to worry unnecessarily.
- Articles should be based on good research by competent researchers. That means that the main sources should be the most well-known scientific journals with peer-review to guarantee, for instance, unbiased statistical standards.
- All stories should be checked and evaluated by other scientists, perhaps with different opinions. In controversies all sides should be heard. Scientific doubts should be shown, and this means that every journalist must have a pool of reliable persons to talk to.

But perfection is not always attainable. Many factors in the media world, including the following, can create mistakes and failures in spite of the best intentions and ambitions.

- The difference between different media: many widespread media such as television are developing towards ever faster and shorter reporting – known as the CNN syndrome – where the news is transformed into commercial goods. This tendency supports superficial news reporting in 'black and white', without time to explain and allow for uncertainties. Many pressure-groups, for instance Greenpeace and AIDS activists, have learned to use these aspects of the media and their actions are tailored to fit the demands of the modern television industry. Newspapers have partly fallen into the same trap, with the same superficial and simplified approach, but these media also try to maintain a more qualified type of reporting. This information is more and more directed only to the well educated. This is a growing social class problem that also affects the whole issue of risk information.
- Time-pressure is another important factor. Daily media are created new every day, and it is a constant dilemma that journalists always have a very short time to write articles. That limits the possibilities to talk to all the necessary people and to carry out all the checks.
- Not all risk-writing is done by experienced and specialized journalists. Media are a news-factory that works all the time, and much of the important newswork is done by so-called general journalists who handle a lot of different matters and cannot be expected to know everything.
- The communication between the journalist and the scientist can easily go wrong. It is a meeting between two separate subcultures with totally different traditions, and good communication demands a respect that unfortunately is seldom there. The scientist should try to avoid manipulating and talking on his or her own behalf, and give the whole picture.

What is the outcome of media reporting on risks? Well, we all know the great discrepancy between the experts and the public in risk perception. A very popular explanation even among some experts is to blame the media for bad and wrong reporting, but that is an oversimplification. For instance, the latest study in Sweden on attitudes to AIDS shows that in 1994 – after almost 15 years of the AIDS epidemic – 28% of the Swedish people still thought that the HIV virus could be spread by insects and 16% from public toilets, even though these misconceptions have never appeared in the media – if anything, the media have tried to correct them.

In a big campaign in California recently, researchers used media – both articles and advertisements – to encourage people to eat fruit and vegetables five times a day, a common message for cancer prevention. But the campaign failed totally. No effect could be observed, although the requested behavioural changes were relatively minor.

Information and knowledge are important, but the public's views and reactions are decided by many other things, for instance the so-called outrage phenomenon – the fact that people choose what they read and believe in. They have a tendency to look for facts that support their existing beliefs. However, the journalistic challenge is, of course, to continue to give proper information, and maybe we have to accept the fact that people have a proportion of irrationality: this may be a part of being human.

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Inhibition of biotransformation by nitric oxide (NO) overproduction and toxic consequences

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Abstract

Hepatic nitric oxide (NO) biosynthesis is induced by local or systemic inflammation. The highly reactive NO radical binds to prosthetic iron groups such as heme or iron-sulfur clusters leading to either activation or inhibition of enzymes such as guanylate cyclase, cyclooxygenase and aconitase. It has been known for years that NO also binds to the heme moiety of cytochrome P450s (CYP) with high affinity. However, it was demonstrated recently that binding of NO to CYPs also inhibits their enzymatic activity. This is true for exogenously applied as well as for endogenously synthesized NO. Suppression of CYP-dependent metabolism, which is a major problem of inflammatory liver diseases, can be significantly reversed by inhibition of NO synthesis in vivo under experimental conditions. We investigated whether these findings are applicable as a novel therapeutic principle in severe inflammatory liver dysfunction.

Keywords: Nitric oxide; Inflammation; Cytokines; Liver; Cytochromes P450

1. Introduction

The liver plays a central role in the response of the organism to inflammatory stimulation. At the beginning, during the so-called acute-phase response, hepatic metabolism is activated to produce various proteins and nutrients, which generally support the body's defense systems. However, in the late course of severe inflammatory diseases, specifically in septic states, the metabolic capacity of the liver is progressively impaired and may end in liver dysfunction in the context of multiple organ failure.

As a novel aspect of liver metabolism during inflammation, increased nitric oxide (NO) biosynthesis was described in hepatocytes as well as in non-parenchymal liver cells [1]. In vitro studies demonstrated that bacterial toxins, such as endotoxin, and proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interferon gamma (IFNγ) are the most important inducers of NO biosynthesis in liver cells [2]. In hepatocytes, the presence of all 4 of these inducers is required for maximal NO production due to a complex mechanism of transcriptional regulation of the responsible enzyme, the inducible nitric oxide synthase (iNOS) [3]. Induction of the iNOS can be suppressed by glucocorticoids and other mediators, especially by growth factors (Table 1). Stimulation of iNOS transcription followed by massive NO production was reported in various species,

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Table 1
Transcriptional regulation of hepatocellular iNOS

Inducers	Suppressors
TNFα	Glucocorticoids
IL-1	TGFα/β
IFNγ	HGF
LPS	EGF

including rodents as well as humans [4,5]. Regulation of NO biosynthesis on the transcriptional level and the level of cofactor supply for enzymatic activity appears to be similar in all species studied so far.

In vivo induction of hepatic NO synthesis was reported in rodents following injection of inactivated *corynebacterium parvum* and malaria sporozoites as well as in endotoxemia and gram-negative sepsis. Inflammatory diseases which are accompanied by hepatic NO biosynthesis are not yet well defined in humans. Preliminary results suggest that iNOS expression may yield a sufficient concentration of NO to fight off viruses, bacteria, and parasites, and may play a role in other situations as well, such as rejection episodes following liver transplantations [1].

2. Pathology and pathophysiology of NO biosynthesis in the liver

In animals treated with *corynebacterium parvum* and lipopolysaccharide (LPS), inhibition of NO biosynthesis resulted in liver damage as indicated by elevation of liver-specific intracellular enzymes in the serum [6]. Histologic sections showed microvascular thrombosis as a possible explanation for these effects. Further studies demonstrated that NO and cyclooxygenase products interact in preventing thrombus formation under these inflammatory conditions [7]. It was also demonstrated that the protective role of NO is in part based on the reaction of NO with oxygen radicals, since scavengers of superoxide and hydroxyl radicals were able to mostly prevent the damage caused by inhibitors of NO synthesis [8].

Using a model of acute murine endotoxemia other investigators showed a NO-mediated upregulation of hepatic protein synthesis [9]. This phenomenon is most likely due to the induction of the acute phase response of the liver which seems to be supported by an increase of perfusion. In this context, it is well established that NO regulates microvascular perfusion, which is the basis for appropriate organ function. It was demonstrated that blood flow is profoundly enhanced by endogenous NO synthesis in the liver as it is in many other organs [10].

Additionally, most in vitro investigations found suppressive effects of NO on metabolic performance of liver cells. For instance, the induction of NO synthesis in hepatocytes is accompanied by a profound inhibition of total cellular protein synthesis [11]. The levels of mRNA of secretory proteins, such as albumin and fibrinogen, were found not to be changed by NO while the concentration of these proteins in the culture supernatants was substantially decreased when the cells were exposed to exogenously applied NO. This indicates a posttranscriptional mechanism by which NO inhibits the synthesis of these proteins [12]. The exact molecular basis of this effect of NO still remains to be uncovered. These data indicate that NO is a substance with many different facets which might produce an overall picture that is different from specific effects on single metabolic pathways.

3. Biochemistry of NO-mediated effects on liver function

The highly reactive NO radical avidly binds to many different molecules and structures which are present in a subcellular environment (Table 2). This very characteristic behavior of radicals is responsible for the short half-life of NO, which is in the range of a few seconds in biological solutions, and the plethora of biological effects of NO [13]. So far, most of the effects of NO in terms of regulation of metabolic pathways were shown to be due to binding to prosthetic iron groups.

Using electron paramagnetic resonance (EPR), it was demonstrated that hepatocyte NO biosynthesis predominantly leads to the formation of iron-nitrosyl compounds [14]. The EPR signal was found to be very similar to the one

Table 2
Molecular basis of biological effects of NO

Chemical reaction	Biological effects
Binding to prosthetic iron groups(heme, iron-sulphur clusters)	Modulation of enzyme activity, generation of methemoglobin
Binding to thiols	Alteration of the functional capacity of specific proteins, transport of NO
Deamination	Destruction of DNA
Reaction with other radical species	Neutralization, enhancement of toxicity

generated by NO-binding to spinach ferredoxin, which contains 4Fe-4S clusters. However, since EPR signals under these conditions represent a summation of many different signals, other forms of NO binding may not have been identified. Furthermore, the signal was detectable in the cytosolic fraction of the cells only, suggesting that there is no NO-mediated inhibition of enzyme complexes of the mitochondrial electron transport chain, which do contain iron-sulphur clusters [15], rather than NO reactions with scavenger molecules such as metallothionein, which is thought to protect the electron transport chain [16].

However, many biological effects of NO are based on binding to prosthetic heme groups. The most prominent example of a heme-containing enzyme, which is regulated by NO, is the soluble guanylate cyclase. NO-dependent cGMP formation was demonstrated in hepatocytes just as in many other cell types [17]. Other examples of hemoprotein activities regulated by NO include cyclooxygenase [18], lipoxygenase [19] and catalase [20]. While the influence of NO on cyclooxygenase and lipoxygenase activity may be important for the production of eicosanoids in the liver by Kupffer cells [21], the inhibitory effects of NO on catalase activity have to be analyzed in situations where detoxification of oxygen radicals is important in the liver.

4. Inhibition of cytochrome P450 activity by NO

A specific family of heme-thiolate enzymes, the cytochrome P450 (CYP) enzymes, play the key role in biotransformation. It has been known for decades that NO binds to the heme moiety of CYPs, and which was used as a spin label probe for CYPs [22]. However, only recently it was reported that treatment of these enzymes with exogenously applied NO inhibits their catalytic activity [23]. Using microsomal preparations and purified CYPs it was also demonstrated that various CYP-dependent reactions show differences in the susceptibility towards the inhibitory effects of NO. Most interesting was the observation of 2 distinct phases of CYP inhibition by NO [23]. The first phase was characterized by reversible, but almost complete cessation of catalytic activity, followed by a second irreversible phase characterized by a varying extent of recovery of activity. These findings led to the suggestion that NO may inhibit CYP enzyme activity by 2 distinct mechanisms. One may be binding of NO to the heme group in the catalytic center of these enzymes. This notion is supported by a study of Khatsenko et al. [24] who demonstrated characteristic changes in absorption spectra which indicate that NO binds to the ferrous as well as to the ferric state of CYP enzymes. Further support of this hypothesis was presented by Lancaster et al. [20] who found microsomal heme loss and consequently an increase in heme-oxidase activity in isolated hepatocytes when NO biosynthesis was induced.

In our own laboratory, we produce genetically engineered cell lines, which constitutively express specific CYP enzymes [25]. The cell lines are derived from V79 Chinese hamster fibroblasts which do not express any endogenous CYP or NOS activity. Therefore, they are ideal tools for studying the effects of NO on CYP activity. In a first set of experiments, we tested cell lines expressing rat and human CYP1A1 and CYP1A2 and found that these enzymes were inhibited by

NO donors SNP and SNAP in a concentration-dependent manner [26]. It was also demonstrated that CYP1A1 was more sensitive towards the inhibitory effect of NO than CYP1A2 in both species. Further experiments focused on the effects of endogenously produced NO on CYP-dependent pathways in hepatocytes. Under the influence of NO biosynthesis, benzo[*a*]pyrene turnover, which reflects the activity of the CYP1A family, was dramatically reduced to almost unmeasurable levels. The addition of N^G-monomethyl-L-arginine, a competitive inhibitor of NO synthesis, led to a significant increase in CYP activity. Western blot analysis demonstrated that the concentrations of CYPs per mg total protein were only marginally decreased by NO synthesis. This is most probably the result of the suppressive effect of NO on total protein synthesis [12]. In contrast, Northern blot analysis revealed a significant decrease of CYP mRNA expression in hepatocytes, which were induced to produce NO. Summarizing these results, it seems that NO has an immediate direct effect on CYP catalytic activity and a secondary long-term effect which suppresses CYP activity by gene inhibition.

As mentioned above, isolated biochemical effects of NO on isolated hepatocytes cannot necessarily be extrapolated into changes of complex pathways of liver function. However, using an experimental model of endotoxemia, Khatsenko et al. were able to confirm the in vitro data of NO-mediated CYP inhibition under in vivo conditions [24]. The authors demonstrated a significant correlation between inhibition of CYP activity and induction of NO biosynthesis in rats treated with i.p. injections of LPS. NO synthesis as well as suppression of CYP activity were largely prevented when these animals received N^{ε}-nitro-L-arginine methyl ester prior to LPS administration. However, this experimental setup is not perfectly relevant in terms of potential clinical applications. Since in the clinical situation the inflammatory process will almost always precede a therapeutical intervention we tested the effect of N^G-monomethyl-L-arginine after the induction of NO biosynthesis in the model of *corynebacterium parvum* injection. Our preliminary results demonstrate a significant improvement of CYP-dependent metabolic reactions as determined by benzo[*a*]pyrene hydroxylation.

Although these data imply an important role of NO in the regulation of CYP-dependent metabolic processes in the liver, future studies have to clarify whether this mechanism is indeed relevant for the clinical situation. If this is the case, novel therapeutic strategies may be the consequence.

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Nitric oxide (NO) protects against cellular damage by reactive oxygen species

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Abstract

Since the discovery of nitric oxide (NO) as an endogenously formed radical, its effect on numerous physiological processes has been intensively investigated. Some studies have suggested NO to be cytotoxic while others have demonstrated it protective under various biological conditions. Though NO shows minimal cytotoxicity to a variety mammalian cell cultures, it does modulate the toxicity of some agents such as reactive oxygen species. Often, NO is generated in the presence of these reactive oxygen species in response to foreign pathogens or under various pathophysiological conditions. We will show that NO can play a protective role under oxidative stress resulting from superoxide, hydrogen peroxide and alkyl peroxides. It was found by measuring the time-concentration profiles of NO released from various NO donor compounds that only μM levels of NO were required for protection against the toxicity of these reactive species. It was found that there are several chemical reactions which may account for these protective effects such as NO preventing heme oxidation, inhibition of Fenton-type oxidation of DNA, and abatement of lipid peroxidation. Taken together, NO at low concentrations clearly protects against peroxide-mediated toxicity.

Keywords: Nitric oxide; Oxidative stress; Oxygen radicals

1. Introduction

Reactive chemical intermediates derived from various substances have been invoked as causative agents in many toxicological mechanisms. Oxygen free radicals are thought to be the causative agents in a wide variety of diseases and degenerative states [1]. Yet, immune surveillance produces these reactive molecules to combat foreign pathogens. The primary mechanism to form these reactive intermediates is often referred to as a Fenton-type reaction which is the reaction between hydrogen peroxide and redox active metals to produce powerful oxidants which can oxidize a wide number of important biomolecules.

$$M + H_2O_2 \rightarrow M{=}O,\ M(H_2O_2) \text{ and/or } OH\cdot \quad (1)$$

These oxidation reactions are thought to be the genesis of many diseases [2].

Recently, the discovery that the molecular free radical, nitric oxide (NO), is endogenously

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formed in biological systems regulating a wide variety of physiological functions has lead to the investigation of the role this molecule plays in various toxicological mechanisms [3–5]. It has been known for years that this molecule forms various reactive nitrogen oxide species (RNOS) in the presence of oxygen which can be deleterious to biological systems [6]. Intermediates formed in the NO/O_2 reaction (sometimes referred to as the autoxidation of NO) can oxidize or nitrosate substrates which can lead to alteration of macromolecules such as enzymes and DNA [7]. In addition to these reactive species formed from the autoxidation, NO reacts with the oxygen radical species, superoxide, at near diffusion control to form the potent oxidant [8], peroxynitrite, which can chemically modify many biological molecules. This mechanism via peroxynitrite formation has been suggested to enhance the cytotoxicity of superoxide [9–11].

Contrary to the deleterious effects of RNOS formed from either NO/O_2 and NO/O_2^-, NO possesses antioxidant properties. Kanner et al. showed that NO could prevent the destruction of hemoproteins by hydrogen peroxide and abate Fenton-type oxidation reactions [12]. Several studies have suggested that NO can act as a chain breaking antioxidant in lipid peroxidation reactions and play a beneficial role in diseases such as atherosclerosis [13,14]. So is NO good or bad? The answer may depend on where and when these intermediates are formed. This report will discuss some of the protective effects of NO with respect to oxygen free radicals and offer some insight as to where this protective effect might occur in vivo. We will review NO's effect on hydrogen- and alkylhydro-peroxide-mediated cytotoxicity and what effect this molecule has on the superoxide generating system, hypoxanthine/xanthine oxidase (XO)-mediated toxicity.

2. NO protection against hydrogen peroxide toxicity

It has been known for a number of years that chemical species derived from the reduction of oxygen, i.e. hydrogen peroxide and superoxide, cause cell death in both eukaryote and prokaryote organisms. The reaction of hydrogen peroxide with various redox active metal complexes leads to formation of powerful oxidants such as hydroxyl radical or metal-oxo intermediates (Eq. 1). These oxidants can oxidize DNA which ultimately results in cell death [15]. Since intermediates derived from NO can also alter biomacromolecules, it would be assumed that the presence of NO might enhance the toxicty of reactive oxygen species (ROS). We therefore decided to address this question by using a chemical approach.

One of the difficulties in determining the effects of NO on various biological systems is the method of delivery. The enzyme nitric oxide synthase (NOS) can continuously generate NO for prolonged periods of time in vivo. Duplicating the enzymatic production of NO chemically is challenging. NO has a limited lifetime in aqueous solution due to its reaction with oxygen [16], hence simple addition of NO or any other RNOS may not represent the physiological condition. Recently, a variety of NO-donor compounds have been used to simulate NO formed in vivo. Sodium nitroprusside and SIN-1 have often been used as NOS mimics. Despite their vasodilatory activity, they do not release significant levels of free 'NO' [17]. *S*-nitrosothiol complexes have been used which do release detectable NO levels, however, the rate of release differs between media and the chemical kinetics of NO release is still poorly understood [18]. A series of compounds known as the NONOates ($RR'N-[N(O)NO]$) spontaneously release NO in a predictable manner [19,20]. By varying the nature of the alkyl group, the release of NO can be varied from as short as 1 min to as long as 3 days [20]. These properties made them ideal to study the effects of NO on oxygen radical toxicity.

Exposure of Chinese hamster V79 cells to varying concentrations of hydrogen peroxide resulted in increasing cell death as assayed by clonogenic methods. Exposure to diethanolamine (DEA)/NO ($Et_2N[N(O)NO^-Na^+]$) (NONOate with half-life of 2.1 min) results in no appreciable toxicity by itself [21], in fact exposure of V79 cells to a variety of 1 mM NO-donor compounds

or a bolus of NO showed little or no toxicity [22]. However, exposure to 0.1 mM DEA/NO with 1 mM H_2O_2 resulted in abatement of the hydrogen peroxide toxicity. The decomposition products of DEA/NO, diethyl amine and nitrite, afforded no protection against hydrogen peroxide-mediated toxicity, in fact sodium nitrite enhanced cytotoxicity. It was concluded that NO released from DEA/NO was protective against hydrogen peroxide toxicity [21,22].

3. NO protection against alkyl hydroperoxides

These studies were further extended to include alkyl hydroperoxides which are thought to mediate their toxic effects at least in part via the destruction of lipid membranes. The cumene and *t*-butyl alkyl hydroperoxides were exposed to Chinese hamster V79 lung fibroblasts cells for 2 h. A dose-dependent increase in cell kill was observed. Addition of DEA/NO resulted in no protection contrary to that observed with hydrogen peroxide [23]. Since DEA/NO liberates NO within the first few minutes of exposure, the NONOate, PAPA/NO [$(NH_3^+(C_3H_6)$-$(N[N(O)NO]^-C_3H_7)$), $t_{1/2}$ 15 min at 37°C, pH 7.4) was chosen which can release NO for over an hour. Exposure of PAPA/NO by itself resulted in little or no toxicity after a 2-h exposure time. When either cumene or *t*-butyl hydroperoxide was exposed with 0.1 M PAPA/NO, near complete protection was observed [23]. This suggests that NO can abate the toxicity of alkyl hydroperoxides.

Why did PAPA/NO protect while DEA/NO did not despite 10 times more NO being released from DEA/NO? This suggested that the quantity of NO exposed to cells was not as important as the duration of NO formation [23]. As will be discussed below the NO time profile is important to understanding the mechanism of these protective effects.

4. NO protection against XO

The near diffusion controlled reaction between superoxide and NO to form the powerful oxidant, peroxynitrite [8], has been suggested as a deleterious mechanism.

$$NO + O_2^- \rightarrow OONO^- \quad (2)$$

Synthetically generated peroxynitrite can oxidize a number of biologically important molecules. Since the biological formation of this species would result from a separate formation of superoxide and NO, we decided to examine the effects of NO in the presence of enzymatically O_2^- generating on cell toxicity. When V79 cells were exposed to hypoxanthine (HX) and XO for various time intervals, there was an increase in cell kill with time of exposure. Using 2 NONOates, SPER/NO ($t_{1/2} = 39$ min) and DEA/NO ($t_{1/2} = 2.1$ min) marked protection was observed similar to that seen for hydrogen peroxide [21]. It was concluded that NO protected cells from XO cytotoxicity [21]. Furthermore, when the toxicity of several reactive molecules, NO, RNOS, O_2^-, and hydrogen peroxide were compared, it was concluded that peroxides were far more toxic to cells than these other intermediates [21]. In fact, peroxides were 100 times more toxic than NO or RNOS. In addition, these experiments suggest that despite the probable formation of extracellular peroxynitrite, NO is still protective against the toxicity by species derived from the enzymatic reduction of oxygen.

5. Effects of various NO donor complexes on ROS

The electrochemical measurement of NO released from the NONOates has given insight into the importance of temporal considerations with respect to NO's antioxidant properties [23]. Several studies have used other NO donor compounds which have been thought to involve the chemical delivery of NO to biological systems. For instance sodium nitroprusside and SIN-1 have been often thought to represent NO chemically. However, when electrochemical techniques were used to detect NO released, no appreciable NO was detected from either of these com-

pounds. When these agents are exposed together with hydrogen peroxide to V79 cells, both agents enhanced peroxide toxicity contrary to other NO releasing compounds. Another class of NO donor compounds are the *S*-nitrosothiol compounds. When either *S*-nitrosoglutathione or *S*-nitroso acetylpenicillamine were exposed together with hydrogen peroxide to V79 cells, partial protection was observed (submitted). Electrochemical analysis of the NO release profile revealed a small flux of NO sustained throughout the peroxide and XO exposure. These results support the notion that continuous small fluxes of NO as low as μM can afford protection to 1 mM hydrogen peroxide. In addition, it warns about overinterpretation of data equating NO effects with the chemical generating system.

6. Mechanistic considerations of NO protection

It is clear that, on a chemical level, NO can protect against peroxide-mediated toxicity. One of the primary targets of peroxide-derived oxidants is DNA. Chemical modification of DNA by

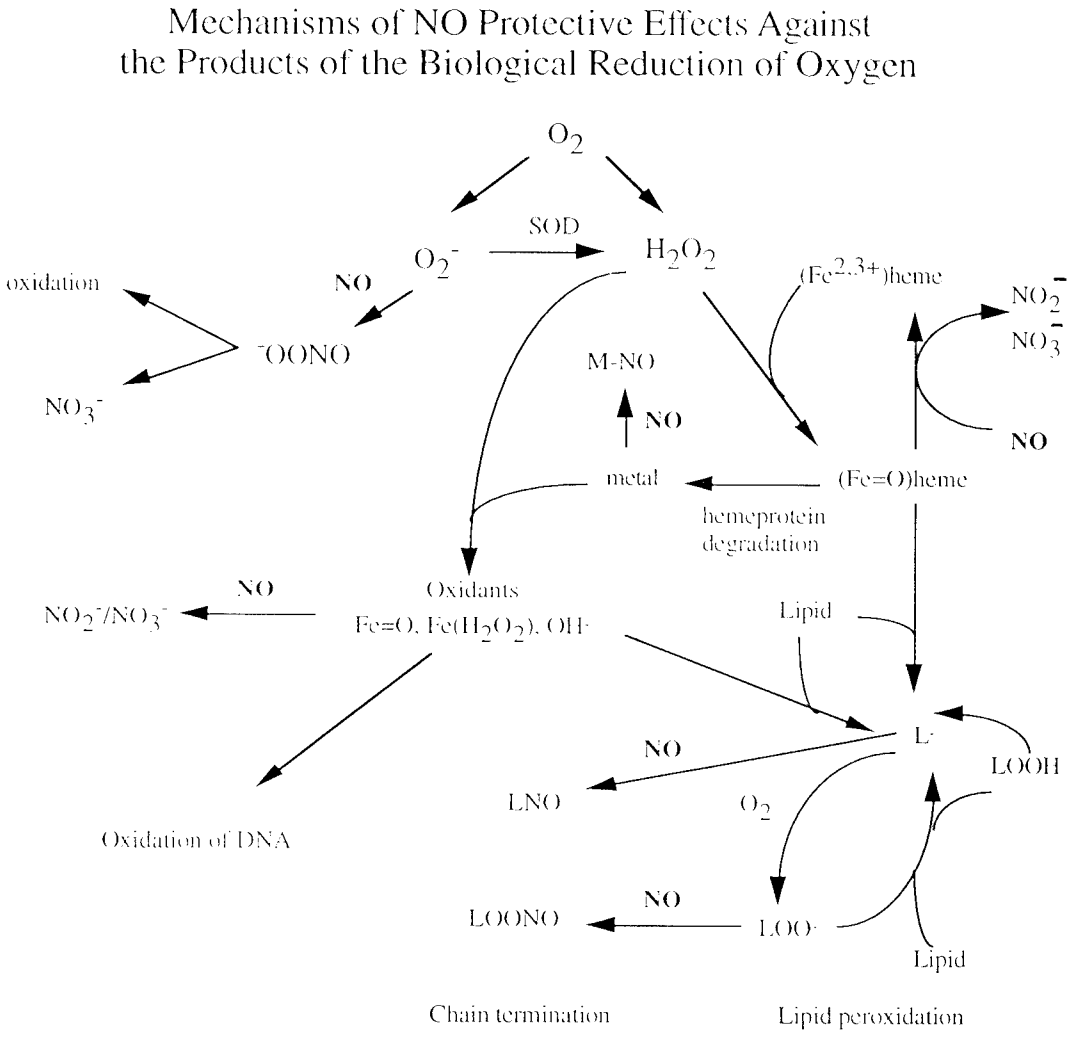


Fig. 1. Mechanisms of NO protective effects against the products of the biological reduction of oxygen.

oxidants can result in a variety of lesions such as single and doubled stranded breaks. These modifications are presumed to be one of the toxic events in the mechanism of peroxide-mediated toxicity. We decided to examine the effect of NO on ferrous ion/hydrogen peroxide-mediated strand breaks of DNA. Supercoiled DNA plasmid was exposed to ferrous ion/hydrogen peroxide. This treatment resulted in extensive single and double strand breaks. However, when the same experiment was done in the presence of DEA/NO or authentic NO, complete protection was observed [24]. These results clearly suggest that the presence of NO can abate the Fenton-type oxidative damage to DNA. It appears that the presence of NO can either prevent the formation of, or scavenge Fenton-type oxidants which affords protection.

Another mechanism which is thought to play a role in the cytotoxic mechanism of peroxide is lipid peroxidation. Alkyl hydroperoxides are thought to mediate, at least in part their cytotoxic action via lipid peroxidation. As discussed above, the presence of NO clearly abates their toxicity. Several studies have reported that NO can abate lipid peroxidation where it was proposed that NO can intercept various lipid radicals forming ROONO adducts which results in termination of the chain propagation reactions (Fig. 1) [13,14]. It has been suggested that these species represent a chain termination step thereby limiting the extent of lipid peroxidation.

There has been some discussion that the reaction between peroxide and ferrous ion is too slow to account for the peroxide exerting significant toxicity in vivo. However, the reaction rate constant between metalloproteins such as hemoproteins and can be $>10^7$ M^{-1} s^{-1} (unpublished result). This suggests that one of the initial targets of peroxide in cells would be these redox active metal centers. For instance, the reaction between myoglobin and peroxide results in a ferryl complex. This hypervalent complex can either facilitate lipid peroxidation [13,14], become covalently altered to form a redox catalyst capable of forming additional peroxide, or decompose releasing free iron into the cell increasing the Fenton-type catalysts [25]. Kanner et al. showed that the ferryl intermediate generated reacts with NO to form the ferric species [12]. This restoration of the native oxidation state prevents the deleterious consequences of this hypervalent species and thereby provides protection.

Collectively these data demonstrate that NO can protect cells from the deleterious effects of peroxide and the resulting ROS formed. It appears that at a chemical level NO has protective properties. Studies utilizing NOS inhibitors in vivo showing protection may suggest that there are physiological mechanisms rather than chemical which could be important as well.

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Toxicology Letters

Tolerance against tumor necrosis factor α (TNF)-induced hepatotoxicity in mice: the role of nitric oxide

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Abstract

D-Galactosamine-sensitized mice challenged with tumor necrosis factor α (TNF) developed severe apoptotic and secondary necrotic liver injury as assessed by histology, measurement of cytosolic DNA fragments and determination of liver-specific enzymes in plasma. Pretreatment of mice with interleukin-1β (IL-1) resulted in elevated levels of nitrite/nitrate in serum and rendered mice insensitive towards TNF toxicity. Pharmacological doses of the nitric oxide (NO) donor sodium nitroprusside (SNP) also conferred complete protection against TNF toxicity, suggesting a possible link between IL-1- and NO-induced protection. However, NO-synthesis inhibition by N^G-monomethyl-L-arginine failed to abrogate IL-1-induced tolerance against TNF toxicity. We conclude that IL-1 and NO protect against TNF-induced liver injury through distinct pathways.

Keywords: Interleukin-1; Sodium nitroprusside; Liver injury; Necrosis; Apoptosis; Galactosamine

1. Introduction

Tumor necrosis factor α (TNF) has been recognized as one of the key inflammatory mediators of bacterial lipopolysaccharides (LPS) and has been shown to mediate lethal shock and multi-organ failure. Transcriptional inhibitors such as the liver-specific agent D-galactosamine (GalN) or actinomycin D (ActD) sensitize mice several thousand-fold towards LPS or TNF [1]. In these models animals develop a relatively selective liver damage which allows quantitative assessment of TNF-toxicity by measurement of liver-specific plasma enzymes [1]. Pretreatment of mice by either LPS, or TNF, or interleukin-1β (IL-1) confers complete tolerance to an otherwise lethal second challenge with LPS or TNF. Tolerance was suggested to be due to one or several protective proteins synthesized within the liver [2].

Circumstantial evidence suggested the involvement of nitric oxide synthase (NOS) in the development of tolerance towards LPS- or cytokine-induced toxicity. NO was shown to be produced in considerable amounts by murine hepatocytes stimulated with cytokines [3]. Moreover, it was shown that endogenously produced NO reduced endotoxin-induced lethality as well as hepatic or renal organ damage [4–6]. Since

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IL-1 is both a strong inducer of NOS [7] as well as a potent agent for induction of tolerance [2], we investigated whether there might exist a causal relation between IL-1-mediated NO formation and tolerance to TNF-induced liver damage in GalN-sensitized mice.

2. Materials and methods

2.1. Animal experiments

Male BALB/c mice from the animal house of the University of Konstanz, Germany, received humane care in compliance with the legal requirements in Germany. All substances were administered in pyrogen-free saline. Sodium nitroprusside (SNP, 1.7 mg/kg, Fluka, Buchs, Switzerland) and GalN (700 mg/kg, Roth, Karlsruhe, Germany) were injected i.p. in a volume of 200 μl, rhuIL-1β (10 μg/kg, a gift from Dr. K. Vosbeck, Ciba Geigy, Basel, Switzerland), rmuTNFα (10 μg/kg, a gift from Dr. Adolf, Bender & Co, Vienna, Austria) and N^Gmonomethyl-L-arginine (NMMA, 175 mg/kg, Sigma Chemical Co., St. Louis, MO) were injected i.v. in a volume of 300 μl. Animals were sacrificed by cervical dislocation 5 or 8 h after challenge and blood was withdrawn by cardiac puncture into heparinized syringes.

2.2. Histology and DNA fragmentation

Livers were perfused for 10 s as described [8] before they were excised. One slice of the large anterior lobe was immediately immersed in 4% formalin and imbedded in paraplast. Sections (2–3 μm) were stained with hematoxylin/eosin. The remaining parts of the liver were homogenized. The homogenate was centrifuged at $13\,000 \times g$ for 20 min. The supernatant (final dilution 1250-fold) was used to measure DNA fragmentation with an ELISA kit (Boehringer, Mannheim, Germany) as described [8].

2.3. Cell culture and incubation conditions

Hepatocytes and non-parenchymal liver cells were isolated from 12-week-old male BALB/c mice by a two-step collagenase perfusion method and cultured as described [8].

2.4. Enzyme assays

Activities of alanine-aminotransferase (ALT) in plasma were determined according to [9].

2.5. Nitrite determination in cell culture supernatants

For determination of nitrite release, cells were incubated for 20 h in RPMI 1640 medium without phenol red. Culture supernatants were assayed for nitrite according to the Griess reaction.

2.6. Nitrite determination in serum

Nitrite in serum was measured essentially according to the method of Misko et al. [10], using the Griess assay.

2.7. Statistics

Data from in vivo experiments are given as means $\pm$ S.E.M., all other data as means $\pm$ S.D. Data for transaminases and DNA fragmentation were analysed by non-parametric analysis of variance (Kruskal-Wallis). Statistical analysis of serum nitrite/nitrate concentrations (Table 1) was done using the parametric Waller-Duncan-Test. $P < 0.05$ was considered to be significant.

3. Results

3.1. IL-1-induced tolerance against GalN/TNF-mediated liver damage

Intravenous administration of 10 μg/kg rmuTNFα to GalN-sensitized mice induced severe necrotic liver damage as determined by elevated plasma levels of ALT 8 h after challenge (6260 ± 1010 U/l vs. untreated control: 40 ± 20 U/l, $n = 6$, $P < 0.05$). Liver necrosis was preceded by apoptotic changes. DNA fragmentation was increased 2.9 ± 0.7-fold 5 h after GalN/TNF administration compared to GalN controls. Eight h after challenge DNA fragmentation was increased 3.6 ± 0.4*-fold compared to controls (data $\pm$S.E.M., $n = 3$, $^*P < 0.05$). Histological examination confirmed these findings: 3.5 or 5 h after injection of TNF to GalN-sensitized mice, hyperchromatic nuclear membranes and formation of apoptotic bodies were seen. Eight h after GalN/TNF, numerous apoptotic bodies were identified between necrotic hepatocytes [11].

Table 1
Effect of the NOS inhibitor NMMA on IL-1-induced tolerance against liver injury caused by TNFα in galactosamine-sensitized mice

Pretreatment[a]	Treatment[b]	ALT[c] (U/l)	Nitrite/nitrate[c] (μM)	n
Saline	GalN/TNF	10 130 ± 2400	35 ± 11	5
IL-1	GalN/TNF	22 ± 24*	32 ± 6	5
IL-1 + NMMA	GalN/TNF + NMMA	35 ± 23*	34 ± 10	5
NMMA	GalN/TNF + NMMA	10 380 ± 3370	27 ± 8	5
IL-1	Saline	17 ± 11*	77 ± 14**	5
Saline	GalN	90 ± 9*	36 ± 9	3

[a] Animals were pretreated 4 h before challenge by an i.v. dose of 10 μg/kg IL-1 ± NMMA (175 mg/kg) or by injection of saline.
[b] GalN (700 mg/kg) was administered i.p. simultaneously with TNF (10 μg/kg, i.v.) and NMMA (175 mg/kg, i.v.).
[c] Plasma ALT was measured in U/l 8 h after challenge, levels of nitrite/nitrate in plasma were determined 6 h after pretreatment. Data are means ± S.E.M.
* $P < 0.05$ vs. GalN/TNF control.
** $P < 0.05$ vs. all other groups.
n, number of animals per group.

Pretreatment of the animals with 10 μg/kg rhuIL-1β i.v. 4 h before challenge led to a completely refractory state against TNF-induced cytotoxicity in GalN-sensitized mice, i.e. neither ALT release into plasma nor DNA fragmentation were significantly increased.

3.2. Prevention of IL-1-induced tolerance by inhibition of hepatic transcription

In order to study the role of hepatic biosynthesis in the development of tolerance, the liver-specific transcriptional inhibitor GalN was given together with IL-1 4 h prior to GalN/TNF-challenge. Under this experimental condition we observed severe liver failure 8 h after challenge (ALT: 8440 ± 1940 U/l). Mice treated with IL-1/GalN and challenged 4 h later with GalN alone developed no liver injury. These observations imply active biosynthetic hepatic processes as a requirement for IL-1-induced tolerance.

3.3. Protection against GalN/TNF-induced liver damage by administration of SNP

Liver NOS is one of the putatively protective proteins [4–6] known to be induced by IL-1 [7]. In order to study a possible participation of NO in the development of tolerance, we first tested the efficacy of the pharmacological NO donor SNP against liver damage induced by TNF in GalN-sensitized mice. Pretreatment with SNP (1.7 mg/kg i.p., 15 min before challenge) protected against liver damage induced by GalN/TNF as shown by significantly reduced levels of plasma ALT compared to GalN/TNF controls (110 ± 25 U/l vs. 8470 ± 4120 U/l, $n = 5$, $P < 0.05$). These results indicate that endogenous NO production from exogenous precursors has the potential to protect against TNF-mediated toxicity.

3.4. IL-1-induced endogenous NO production

Since pharmacologically delivered NO provided protection against TNF toxicity in vivo, we checked whether NO was produced endogenously upon treatment of mice with IL-1. Serum nitrite/nitrate levels were elevated 6 h after administration of IL-1 as compared to saline-treated animals (Table 1). In order to identify the source of IL-1-induced endogenous NO production, hepatocyte and non-parenchymal liver cell cultures from IL-1 or saline-treated animals were prepared 4 h after pretreatment. Endogenous NO production was determined by measuring the amount of nitrite released into the culture supernatant. Hepatocytes were left untreated or were further stimulated by incubation with IL-1 (50 ng/ml). In vivo pretreatment with IL-1 lead to an increased ex vivo nitrite production in liver

Table 2
Ex vivo nitrite production by hepatocytes of IL-1-pretreated and control mice

Pretreatment[a] (in vivo)	Stimulus (in vitro)	
	None	IL-1 (50 ng/ml)
Saline	<1[b]	2.8 ± 0.6
IL-1	4.8 ± 1.2	19.8 ± 2.9

[a] Animals were pretreated 4 h before preparation of hepatocytes. IL-1 was given i.v. in a dose of 10 μg/kg.
[b] Hepatocytes (8×10^4 per well) were incubated in RPMI 1640 for 20 h before the amount of nitrite was assayed in the supernatant. Data are means (nmol nitrite/10^6 cells) ± S.D. of triplicate incubations.

cell cultures as compared to hepatocytes from saline-treated control animals (Table 2). This augmented basal release was further enhanced more than 4-fold when these already activated cells were incubated with IL-1 in vitro (Table 2). Additional experiments with the same mice showed that non-parenchymal liver cell cultures prepared from cells by differential centrifugation and plastic adhesion produced less than 5% per cell of the nitrite found in hepatocyte cultures, suggesting that the nitrite determined in the above experiments was predominantly derived from hepatocytes (data not shown).

3.5. No impairment of tolerance by inhibition of NOS in vivo

In order to study the relevance of endogenously formed NO for development of tolerance in vivo, endogenous NO production was inhibited by administration of NMMA (2×175 mg/kg), a competitive inhibitor of NOS, together with IL-1 pretreatment and together with a subsequent TNF challenge. Administration of NMMA caused neither an altered toxicity of TNF in vivo nor any modification of the IL-1-induced tolerance in GalN-sensitized mice (Table 1).

4. Discussion

A large variety of experimental models have been used in order to study possible mechanisms of septic shock and ensuing organ failure. LPS as the primary initiator of a cytokine response or TNF as a distal mediator of LPS toxicity are commonly used to elicit the systemic inflammatory response. However, pretreatment of mice with minute amounts of either one of these agents protects them from a second, otherwise lethal challenge. In the present study we used IL-1 to induce tolerance against TNF challenge in the low-dose model of the GalN-sensitized mouse.

A salient feature of the GalN/TNF model is apoptotic and secondary necrotic liver cell death [11]. We therefore measured cytosolic DNA fragments and hepatocyte-specific enzymes to quantitate organ injury. According to these parameters, IL-1 completely protected mice against hepatic injury induced by TNF. Since the development of tolerance could be completely abolished by co-administration of GalN, which selectively impairs the hepatic RNA synthesis [11] we conclude that IL-1-induced tolerance is an active process requiring hepatic transcription.

One putatively protective protein known to be induced by IL-1 is NOS, an enzyme producing the potent vasodilatator NO. Our finding that NOS activity is increased by incubation of murine liver cell cultures with IL-1 in vitro is in agreement with previous findings showing an induction of NOS mRNA in human hepatocytes under similar conditions [7]. In analogy to the in vitro experiments, treatment of mice with IL-1 in vivo led to elevated levels of nitrite/nitrate in serum and to an increased NOS activity in liver cells ex vivo.

The role of endogenously produced NO in septic shock and other inflammatory models is not yet clarified. In order to study the significance of NO in TNF-induced liver failure in GalN-sensitized mice and to further investigate the role of endogenously formed NO for the development of tolerance, we examined in vivo whether injection of an NO-releasing agent (SNP) could substitute for IL-1 in the protection against TNF toxicity and which effects inhibition of NOS might have on IL-1-induced tolerance. The protection by SNP against TNF-induced hepatotoxicity is consistent with findings in the GalN/LPS model where various vasodilatators prevented liver injury [1]. Though an increased production of NO is probably responsible for the

detrimental fall in blood pressure seen in septic shock [12], beneficial effects of this mediator in septic complications have also been described. For instance, an aggravation of LPS-induced hepatic damage in *C. parvum*-sensitized mice by administration of NMMA, a competitive inhibitor of NOS, was reported [5]. However, we found that NMMA administration at a dose described to block the endogenous NO formation [5] did not abolish IL-1-induced tolerance. This argues against a predominant role of endogenously produced NO in this inflammatory model and against a role of NO in the development of tolerance.

We conclude that IL-1 induces expression and/or release of tolerogenic proteins different from NOS and that increased production of endogenous NO is not the mechanism responsible for tolerance development.

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Toxicology Letters

The role of nitric oxide in cell injury

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Abstract

Nitric oxide (NO) is a pathophysiological mediator with unique signal transducing properties. Signaling mechanisms are categorized as cGMP-dependent or cGMP-independent. Multiple interactions of NO with oxygen, superoxide, and transition metals determine the biological activity. Cyclic GMP-independent responses of NO account for the antimicrobial, the cytostatic, and the cytotoxic capacity of NO. Cytotoxicity is not only directed to harmful cells but also affects the NO-producing cell in a self-destructing loop. For macrophages and pancreatic β-cells (RINm5F), we established NO-mediated apoptotic cell death. Endogenously generated or exogenously applied NO causes DNA cleavage after endonuclease activation. NO-mediated accumulation of the tumor suppressor p53 precedes apoptotic cell death.

Keywords: Nitric oxide; Nitric oxide synthase; NO-donor; Apoptosis; DNA fragmentation; Tumor suppressor p53

1. Nitric oxide formation; nitric oxide synthase and NO-donors

Nitric oxide (NO) is generated by a family of enzymes termed NO synthases (NOS) which utilize arginine as their substrate [1]. For simplistic reasons, NOS isoforms can be categorized by descriptive terms based on the dependence of intracellular calcium transient required for full activity. Constitutive forms are activated by a time-limited cytosolic calcium increase, which leads to the release of NO over several minutes. A cytokine-inducible NOS isoform is expressed in many cells after challenge with immunologic or inflammatory stimuli, producing large amounts of NO, for up to several days [2]. The determinant of isoenzyme activity allows approximation of the classification as a low versus high output system for endogenously generated NO. Multiple transcriptional as well as translational control mechanisms operate to augment or suppress NO formation that allows a fine tuning of NO generation, accumulation, and action [3]. Several NOS inhibitors, like N^G-monomethyl-L-arginine (NMMA), are used to intervene pharmacologically with NO production, that permits a trace back of individual actions to the NO-signaling system.

To study physiology, pathophysiology, and pharmacology of NO, irrespective of NOS involvement, NO-releasing compounds are valuable tools. [4]. All NO-donors preserve NO in their molecular structure and all exhibit biological activity after decomposition. These prodrugs vary considerably in chemical structure,

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stability, and biological activity. Different pharmacokinetic profiles arise in part from differences in bioactivation and enzymatic versus nonenzymatic NO-release. Examples are organic nitrates, sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), *S*-nitrosothiols (i.e. *S*-nitrosoglutathione, GSNO or *S*-nitrosocysteine, CysNO), and compounds containing the N(O)NO$^-$ functional group (i.e. spermine-NO).

2. NO signaling

After release, NO$^\bullet$ is susceptible to both oxidation and reduction [5]. The formation of NO^+ (nitrosonium ion) and NO^- (nitroxyl anion) as primary reaction products allow secondary target interactions that are fundamental to the understanding of the biochemistry and biological function of NO. In biological systems NO reacts with oxygen, superoxide, and transition metals. Reaction products – NO_x, peroxynitrite (OONO$^-$), and metal-NO adducts, respectively – support additional reactions through their interactions with targets via redox and additive chemistry. The intracellular prevalence and reactivity of thiols (RSH/RS$^-$) over other nucleophils support nitrosative reactions (i.e. NO^+-related chemistry) for *S*-nitrosothiol (RS-NO) formation [6]. Thus, receptors, ion channels, enzymes, or transcription factors containing either transition metals or thiols located at the active or allosteric sites are the essential components of the NO signaling system.

3. Physiology and pathophysiology

For physiology, the interaction of NO with the heme regulatory subunit of soluble guanylyl cyclase is most important. The endothelium derived relaxing factor (EDRF)-like function is the key transducer of the vasodilator message from the endothelium to vascular cells. Activation of guanylyl cyclase, formation of cGMP, and concomitant protein phosphorylation forms an inter- and intracellular NO-responsive regulatory network [7]. For the pathophysiological and cytotoxic action of NO, the radical nature of NO$^\bullet$ is not sufficient to explain its lethal activity. Cytotoxicity is not only directed to invading bacteria, microorganisms, and tumor cells as a first line of the unspecific immune defense system, but also affects NO-producing cells like macrophages in a self-destructive loop (for references, [8]). Examples of toxic actions of NO are major neurodegenerative diseases [9] or pancreatic β-cell destruction, linked to type I diabetes mellitus (for references, [10]). Mechanistically, the diffusion-limited reaction of NO$^\bullet$ with superoxide known to generate OONO$^-$, inhibition of FeS-enzymes like the Krebs cycle aconitase, complex I and II of the mitochondrial respiratory chain, or ribonucleotide reductase, deregulation of poly-ADP-ribosyltransferase, and energy depletion are discussed as a likely scenario for cell death (for references, [6,8,11]). However, as the toxicity of NO is dependent on the biological milieu, reactions of NO with reactive oxygen species (ROS) may be toxic or protective, depending on the nature of the insult. Exemplified for neuronal cells, the diffusion-limited reaction of NO$^\bullet$ with ROS will generate OONO$^-$, a potentially toxic molecule [12]. To the contrary, NO$^\bullet$ may have protective effects during ischemia reperfusion injury and effects that essentially abrogate ROS-initiated cytotoxic effects on mesencephalic cells or lung fibroblasts [13]. Such opposing effects are even predictable for systems sensitive to redox regulation and may extend to cell necrosis or apoptosis as mechanisms for cell destruction.

4. NO and apoptotic cell death

Apoptosis is a morphologically distinct form of cell death that appears to be common to all multicellular organisms. It is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. Initiation is achieved by a wide variety of stimuli, with the identification of genes positively or negatively involved in the cell-intrinsic suicide program (for references, [14]).

For macrophages (RAW 264.7) and a β-cell line (RINm5F), NO-mediated cytotoxicity occurs through apoptosis rather than necrosis (for refer-

ences, [15,16]. Although pancreatic islet DNA has been proposed as a target for NO, no DNA fragmentation was rationalized. In RINm5F cells, interleukin 1-β (IL-1β)-induced NOS expression clearly preceded DNA laddering, nuclear condensation, and apoptotic body formation. The finding that typical apoptotic features were suppressed by NOS inhibitors support a role for NO-triggered apoptosis in this system [15]. Comparable to RINm5F cells, activation of endogenous NO formation after NOS induction showed apoptotic morphology and exhibited biochemical apoptotic features in RAW 264.7 macrophages [16]. Cytokine-elicited apoptosis was prevented by blocking NOS activity, using NMMA. Thereby we established a link between NO generation and cell destruction. Similar results became apparent after exogenous NO-donor application. A chemically heterogeneous group of NO-donors like SNP, SIN-1, GSNO, and spermine-NO initiated a time- and concentration-dependent biological effect. However, we realized compound specific differences. Initiation of apoptosis is related to the half-life of these compounds and the NO-redox species being released. Generally, GSNO and spermine-NO are the most potent inducing NO-donors, whereas SIN-1 is least active. Addition of lipophilic cGMP analogs, i.e. 8-bromo-cGMP or dibutyryl-cGMP did not result in cell death, excluding the involvement of guanylyl cyclase and the cGMP signaling cascade.

Additional experiments characterized signaling components involved in DNA fragmentation induced by chemically generated NO. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), a commonly used PKC-activating agent, suppressed DNA fragmentation induced by SNP and GSNO. Prolonged incubation of cells with TPA down-regulates certain PKC isoenzymes. However, down-regulated PKC no longer inhibits NO-induced apoptosis. PKC antagonistic data are supported by the notion that PKC inhibitors such as staurosporine or calphostin C sensitize macrophages to NO-induced cell death. Co-incubation of cells with staurosporine and GSNO or SNP resulted in increased fragmentation in each case. Furthermore, cAMP turned out to be a negative modulator of NO-mediated signaling during apoptosis. Lipophilic, membrane-permeable cAMP analogues like 8-(4-chlorophenylthio)-cAMP or dibutyryl-cAMP, dose-dependently decreased SNP, SIN-1, or SNAP-induced DNA cleavage. In an analogy to several other systems, protection was observed in the presence of Zn^{2+}, an inhibitor for the Ca^{2+}, Mg^{2+}-dependent endonuclease, and likely involved in DNA cleavage.

5. The tumor suppressor p53 and NO-driven apoptosis

p53 is a tumor suppressor protein that is important in maintaining genomic integrity. The protein has been suggested to act as a 'guardian of the genome', monitoring the state of cells' DNA. Normally, the half-life of p53 is short, with undetectable or low amounts of the protein in the cytosol. The protein amount is induced to high levels after DNA damage, leading to cell growth arrest. A loss or mutation of p53 is found in high rates in tumor development. High levels of p53 induction in response to DNA damage correlate with arrest at the G1 stage of the cell cycle or in the case of severe DNA damage with apoptosis (for references, [17,18]). Our results using RAW 264.7 macrophages or RINm5F cells established a role of p53 during NO-mediated cell death [8]. Activation of the cytokine/lipopolysaccharide-inducible NOS caused not only massive nitrite accumulation in the cell supernatant but also resulted in p53 accumulation, clearly preceding DNA fragmentation. All apoptotic features, including accumulation of the tumor suppressor p53, were down-regulated by the NOS inhibitor NMMA. This links endogenous NO formation, p53 accumulation, and apoptosis. The involvement of NO during p53 accumulation was further substantiated by using several NO-donors like GSNO, SNP, or spermine-NO. The extracellular applied NO-releasing compounds decompose to release $NO^{\bullet}$ or NO^{+}. Using RAW 264.7 macrophages Fig. 1 shows the time course for p53 accumulation and DNA laddering in response to spermine-NO.

Evidently p53 accumulation, detected by Western blot analysis and quantitative phosphor im-

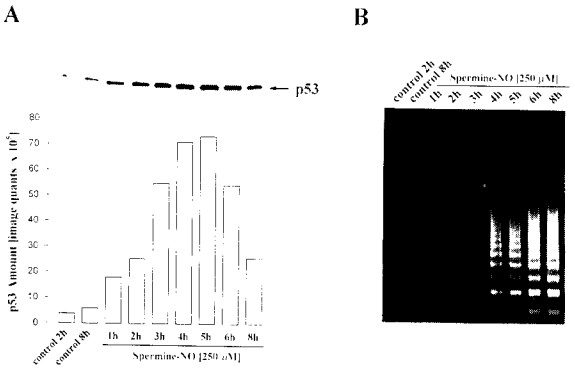


Fig. 1. p53 accumulation and DNA fragmentation in response to spermine-NO. RAW 264.7 macrophages (2×10^7 cells) were incubated with 250 μM spermine-NO for the times indicated. (A) Following the incubation, p53 was immunoprecipitated, detected by Western blot analysis, and quantitated by phosphor imager analysis as described [8]. (B) DNA laddering initiated by spermine-NO was assayed by separation of fragments from intact chromatin, visualized by UV transillumination after agarose gel-electrophoresis. Results are representative of 3 similar assays.

ager analysis, increased after 60 min following supplementation with 250 μM spermine-NO (Fig. 1A). Fig. 1B indicates DNA cleavage into oligonucleosomal fragments. Cleavage becomes visible after 4–5 h, with substantial laddering after 6–8 h. Similar results were obtained in RINm5F cells with the use of SNP [8,15]. The potential of NO to induce p53 accumulation is evident in different cells, regardless of whether it is formed endogenously after NOS induction or generated by structurally different NO-releasing compounds. Northern blot analysis revealed that p53 accumlation does not depend on mRNA production. p53 protein accumulation can be a part of signaling leading to apoptosis either by acting directly on the DNA or by blocking the cell cycle.

6. NO species during cytotoxicity

The multiplicity of biological functions thus far attributed to NO has led to suggestions that various effects might be mediated by other related species instead. A predominant mechanism by which cytotoxicity is thought to occur is through the diffusion-limited reaction of $NO^{\bullet}$ with superoxide to generate peroxynitrite ($ONOO^-$) [19]. However, in some experimental systems like NIH 3T3 fibroblasts the generation of NO protects from superoxide toxicity and vice versa. Fibroblasts incubated for 24 h exhibit a low spontaneous apoptotic fragmentation (Fig. 2). Application of a redox cycling quinone, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) [20] at a concentration of 5 μM causes massive apoptotic cell death. Also, the addition of 2 mM GSNO produces dramatic DNA fragmentation. However, a combination of both substances lowered fragmentation to values below rates seen with each compound alone.

In this case, the forced reaction of NO with reactive oxygen (O_2^-) is protective rather than toxic. Although the experimental design would favour the generation of $ONOO^-$, its action seems not to cause increased damage to fibroblasts. For $ONOO^-$ both cell destructive and protective mechanisms have been reported [12,13]. Similar considerations for opposing ef-

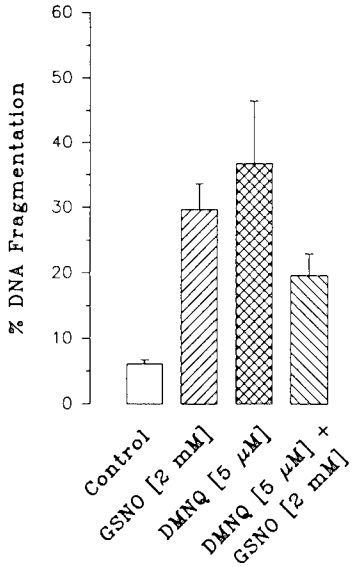


Fig. 2. DNA fragmentation in response to NO and superoxide generating compounds. Fibroblasts (2×10^7; NIH 3T3 cells) were incubated with 2 mM GSNO and 5 μM DMNQ for 24 h, followed by quantitation of the amount of fragmented DNA using the diphenylamine assay. For details see [16]. Mean values ±S.D. of at least 3 separate experiments are given.

fects of NO and related species, including $ONOO^-$, may extend to apoptosis.

Our study clearly showed that NO serves to eliminate apoptotic cell death associated with oxygen species. A protective role of NO under conditions of simultaneous superoxide formation exists, at least for some cells, and may prevent an otherwise toxic oxygen insult. Therefore, the toxicity of $ONOO^-$ depends on specified cellular conditions and can not be addressed in a general way.

The toxicity of NO is influenced by the existing biological milieu. Relative rates of $NO^\bullet$ formation, its oxidation and reduction, the combination with oxygen, superoxide, and other biomolecules will determine the signaling pathway of the radical. This also may provide an answer to the question of how generator cells defend themselves against NO production and lethal effects. It will be interesting to define the versatility of NO-signaling mechanisms in relation to its apoptotic inducing ability and to explore how NO-responsive targets serve both sensory and regulatory roles in transducing a signal. The switch from physiology to pathophysiology, the action of potentially protective and destructive NO species, and the molecular recognition of these balances will be central to the understanding of NO-mediated apoptotic cell death.

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Toxicology Letters 82/83 (1995) 239–245

Toxicology Letters

Sodium channels and $GABA_A$ receptor-channel complex as targets of environmental toxicants

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Abstract

Voltage-activated sodium channels and $GABA_A$ receptor-chloride channel complex are among the most important target sites of various environmental neurotoxicants. Pyrethroids keep the sodium channels open for prolonged periods of time leading to hyperexcitation of the entire nervous system. In rat cerebellar Purkinje neurons and dorsal root ganglion neurons, only about 1% of sodium channel population needed to be modified by the pyrethroid tetramethrin to increase the depolarizing after-potential to the level of the threshold membrane potential for generation of repetitive after-discharges. This concept of toxicity amplification is applicable to other chemicals that go through a threshold phenomenon to exert their effects. The potency of pyrethroids on neuronal sodium channels increased with lowering the temperature with a Q_{10} value of 0.2. The selective pyrethroid toxicity between mammals and insects can be quantitatively explained on the basis of the differences in 5 factors, i.e. the intrinsic sodium channel sensitivity, the sodium channel modification due to temperature difference, the reversibility of sodium channel, the detoxication of pyrethroids, and body size. These 5 factors are multiplied to approximately 2000 which is in the same order of magnitude as that of the difference in LD_{50}. Dieldrin had a dual action on the $GABA_A$ receptor-chloride channel complex of rat dorsal root ganglion neurons. The initial transient potentiation of GABA-induced currents after application of dieldrin was followed by a suppression. Dieldrin-induced potentiation of current was observed only when the γ_2 subunit was present in embryonic kidney cells (HEK-293) transfected with GABA receptor subunits. Dieldrin-induced suppression was observed in the presence and absence of the γ_2 subunit. The dieldrin suppression of GABA-induced currents is deemed directly responsible for hyperactive symptoms of poisoning in animals.

Keywords: Pyrethroids; Dieldrin; Sodium channels; GABA receptor; Selective toxicity; GABA receptor subunits

1. Pyrethroid modulation of sodium channels

It is now well established that the pyrethroid insecticides modify the gating kinetics of voltage-activated sodium channels leading to various hyperactive symptoms of poisoning in animals [1–4]. Previous studies of pyrethroid actions on nerve were conducted mainly with invertebrate nerve fibers and amphibian nerve preparations. The only mammalian preparation used for electrophysiological analyses of pyrethroid action is neuroblastoma cells. However, since neuroblas-

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toma cells are known to be somewhat different from mammalian neurons, it is of crucial importance to use mammalian neurons as material in order to elucidate the mechanism of pyrethroid intoxication. We have started using dorsal root ganglion (DRG) neurons and cerebellar Purkinje neurons of the rat. DRG cells of the rat are endowed with 2 types of sodium channel, one sensitive to tetrodotoxin (TTX) and the other largely insensitive to TTX. The difference in the K_i values for TTX block is as much as 10^5 times [5]. These 2 types of sodium channel also exhibit different sensitivities to other chemicals including lidocaine and chlorpromazine.

1.1. Percentages of sodium channels modified by pyrethroids

Tetramethrin modified both TTX-S and TTX-R sodium channels, but in somewhat different manners [6]. In TTX-S sodium channels, the slow sodium current during step depolarization was increased somewhat by tetramethrin, and a tail sodium current with a slowly rising and falling phase appeared upon repolarization (Fig. 1A). In TTX-R sodium channels, the slow sodium current during step depolarization was markedly increased by tetramethrin, and upon repolarization a large instantaneous tail current was generated and decayed slowly (Fig. 1B). In TTX-S channels exposed to tetramethrin, the tail current developed even after the sodium current during depolarization had subsided. However, in the tetramethrin-modified TTX-R channels, the sodium current during depolarization and the tail current upon repolarization developed and decreased in parallel with each other. The steady-state sodium channel inactivation curve was shifted by tetramethrin in the hyperpolarizing direction in both TTX-S and TTX-R channels. The sodium conductance-voltage curve was also shifted by tetramethrin in the hyperpolarizing direction in both TTX-S and TTX-R channels, and the latter was affected more strongly than the former.

We have developed a method by which the percentage of sodium channels modified by pyrethroids can be measured [6]. The slow tail current associated with step repolarization is

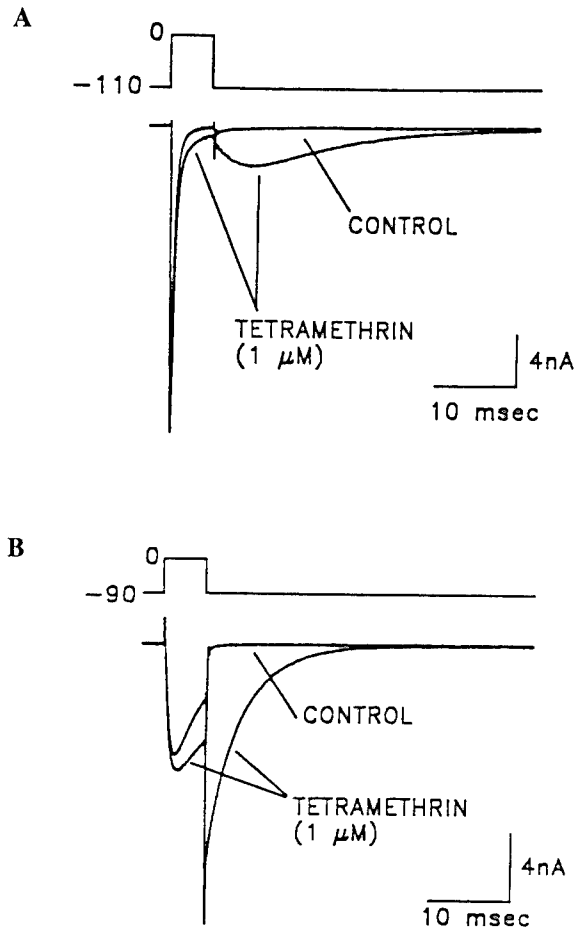


Fig. 1. Effects of tetramethrin on TTX-S sodium current (A) and TTX-R sodium current (B) in rat DRG neurons. A step depolarization to 0 mV was applied from a holding potential of −110 mV (A) or −90 mV (B) in control and in the presence of 1 μM tetramethrin. (Reproduced with permission from Ref. [6]. Copyright 1994 by the American Society for Pharmacology and Experimental Therapeutics).

indicative of the activity of the pyrethroid modified sodium channels, whereas the peak current during step depolarization is a result of the activity of the normal unmodified sodium channels. Thus the sodium conductance upon repolarization can be divided by the peak conductance during step depolarization to calculate the percentage of the modified sodium channels. The percentage of the sodium channels modified by tetramethrin (M) was calculated by the following equation:

$$M = [\{I_{tail}/(E_h - E_{Na})\}/\{I_{Na}/(E_t - E_{Na})\}] \times 100 \quad (1)$$

where I_{tail} is the tail current amplitude obtained by extrapolation of the slowly decaying phase of the tail current to the moment of membrane repolarization assuming a single exponential decay, E_h is the potential to which the membrane was repolarized, E_{Na} is the equilibrium potential for sodium ions obtained as the reversal potential for sodium current and E_t is the potential of step depolarization. The concentration-response data were fitted to the Hill equation:

$$M = M_{max}/\{1 + (K_d/[TM])^h\}, \quad (2)$$

where [TM] and K_d represent the concentration and apparent dissociation constant of tetramethrin, respectively, and h represents the Hill coefficient.

The concentrations of tetramethrin required to modify 1.3% of sodium channels were 10 nM for TTX-R channels and 300 nM for TTX-S channels (Table 1). To modify 12–15% of channels, 100 nM tetramethrin was required for TTX-R channels, whereas as much as 10 μM tetramethrin was required for TTX-S channels. Therefore, the difference in tetramethrin sensitivity between TTX-R and TTX-S sodium channels is as much as 30–100 times. Invertebrate nerve sodium channels, which are sensitive to TTX, are almost equally sensitive to pyrethroids to DRG TTX-R channels.

Whereas the data with rat DRG neurons are highly informative, a question is raised as to how the sodium channels in the brain behave in response to pyrethroids. Cerebellar Purkinje neurons of the rat were found to be endowed with TTX-S sodium channels, which were as insensitive to tetramethrin as TTX-S sodium channels of DRG neurons [7]. The percentages of the sodium channels modified by tetramethrin ranged from 0.6% at 0.1 μM tetramethrin to 25% at 30 μM (Table 1). In order to compare these data on channel modification with repetitive discharges caused by an increase in depolarizing after-potential, current clamp experiments were performed with cerebellar Purkinje neurons. The threshold concentration of tetramethrin to induce repetitive after-discharges was 0.1 μM. Therefore, modification of 0.6% of the sodium channels is enough to cause repetitive after-discharges leading to hyperexcitatory symptoms of poisoning in animals.

Our 'toxicity amplification' theory, which was originally proposed with squid giant axons on the basis of several assumptions [8], is now firmly established in mammalian neurons on the basis of solid experimental data. This concept has important implications to evaluate effective concentrations of toxicants or therapeutic drugs that act via a threshold phenomenon. Take an example in which a slow membrane depolarization caused by channel openings reaches the threshold and induces repetitive discharges. Dis-

Table 1
Percentages of the fraction of sodium channels modified by tetramethrin
Tetramethrin concentration (μM) Cerebellar Purkinje neuron Dorsal root ganglion neuron[a]

Tetramethrin concentration μM	Cerebellar Purkinje Neuron	Dorsal Root Ganglion Neuron*	
	TTX-S (%)	TTX-S (%)	TTX-R (%)
0.01			1.31 ± 0.28
0.03	0		5.15 ± 0.30
0.1	0.62 ± 0.15	0.24 ± 0.10	15.35 ± 0.79
0.3	2.19 ± 0.36	1.25 ± 0.13	35.48 ± 2.70
1	5.75 ± 0.87	3.53 ± 0.66	57.82 ± 2.29
3	13.58 ± 1.35	7.70 ± 1.20	74.85 ± 1.23
10	22.77 ± 2.26	12.03 ± 1.89	81.20 ± 1.57
30	24.73 ± 2.11		

[a] Reproduced with permission from Ref. [6]. Copyright 1994 by the American Society for Pharmacology and Experimental Therapeutics.

charges may be stopped once the slow depolarization is brought down by a drug just below the threshold for excitation. The concentration of the drug for this action will be much less than the EC_{50}. Thus, as far as drugs that act through a threshold phenomenon are concerned, we need to reconsider the traditional pharmacological concept which dictates the comparison of the in vitro EC_{50} value with the drug concentration in the patient serum.

1.2. Temperature dependence of pyrethroid modification of sodium channels

It is well known that temperature has a profound effect on the insecticidal activity of pyrethroids with their potencies increasing with lowering the temperature [3,9,10]. The negative temperature dependence of the insecticidal activity of pyrethroids is due largely to an increase in nerve sensitivity to the insecticide with lowering the temperature [2,9,10]. However, no data are available for mammalian brain neurons and no mechanism underlying the temperature dependence of pyrethroid sensitivity of sodium channels has been elucidated yet.

Both current clamp and voltage clamp experiments were performed with rat cerebellar Purkinje neurons. Tetramethrin at 100 and 300 nM, which modifies 0.62 and 2.19% of sodium channels, respectively (Table 1) induces repetitive after-discharges at low temperatures (15–20°C) but repetitive responsiveness subsided at higher temperatures (30–35°C). Voltage clamp experiments disclosed profound influence of temperature on tetramethrin-induced slow tail currents. While the peak amplitude of tail current was not drastically changed by temperature change, both the rising and falling phases of slow tail current were greatly slowed by lowering the temperature. The temperature-dependent effect of tetramethrin was analyzed with respect to 3 different parameters. The percentages of modified channels by 3 μM tetramethrin and the time constant of the falling phase of tetramethrin-induced tail current were decreased by raising the temperature. The ratios of the charge movement during the tail current to that during the peak current were drastically decreased with increasing the temperature.

The temperature dependence of tetramethrin effect on sodium current can be described by their temperature coefficient Q_{10} which is calculated from the equation:

$$Q_{10} = (X_2/X_1)^{[10/(T2-T1)]} \quad (3)$$

where X_1 is the value of the experimental parameter measured at a low absolute temperature $T1$, and X_2 is that at a high absolute temperature $T2$. The Q_{10} values for the percentage of the sodium channels modified by 3 μM tetramethrin are about the same in the 2 temperature ranges, 0.77 and 0.79 for 20–30°C and 25–35°C, respectively. The charge movement during tail current shows a large negative temperature coefficient, with the Q_{10} values of < 0.22 and 0.18 for 20–30°C and 25–35°, respectively. Thus, the increased and prolonged flow of sodium ions through tetramethrin-modified sodium channels at low temperatures augments the depolarizing after-potential which in turn reaches the threshold for repetitive after-discharges. Although the effects of pyrethroids on the nerve as a function of temperature have been studied using various materials by observing either action potentials or sodium currents, the present study is the first to successfully correlate changes in action potentials and sodium currents in the presence of pyrethroids and as a function of temperature in the same preparation.

1.3. Selective toxicity of pyrethroids

Pyrethroids are much more potent on insects than on mammals. The mechanism underlying this selective toxicity has traditionally been ascribed to the differences in metabolic degradation of pyrethroids between insects and mammals. However, this factor alone fails to explain large differences in LD_{50} values. We have finally come to the conclusion about the mechanism of selective toxicity of pyrethroids by carefully taking into consideration 5 factors. The most important are 3 factors that involve the sensitivity of sodium channels to pyrethroids, the temperature coefficient of action of pyrethroids on

sodium channels, and the reversibility of sodium channels from pyrethroid intoxication.

The difference in each contributing factor between rat Purkinje neurons and invertebrate axons was calculated. First, since there is a 10°C difference in temperature between mammals and invertebrates and also since the Q_{10} value for pyrethroid action on the nerve membrane is 0.2 in both cases, the difference in toxicity ascribed to temperature difference is estimated to be 5. Second, the intrinsic sensitivity of sodium channels to tetramethrin is at least 10 times lower in mammals than in vertebrates. Third, the recovery of sodium channels from tetramethrin intoxication after washing in vitro is at least 5 times faster in mammals than in invertebrates. A fourth contributing factor is enzymatic detoxication of pyrethroids which is estimated to be 3 times faster in mammals than in invertebrates due to temperature difference. A fifth factor is body size; pyrethroids have more chance to be detoxified before reaching the target site in mammals than in invertebrates with an estimated difference of at least 3. Thus, the overall difference in tetramethrin toxicity is estimated to be 2250-fold between mammals and invertebrates which is in the same order of magnitude as the differences in measured LD_{50} values of 500–4500-fold for tetramethrin [11–14].

2. Dieldrin modulation of GABA receptor-channel complex

2.1. Dual action of dieldrin on GABA system

We have embarked on an extensive study of the action of the cyclodiene dieldrin on the $GABA_A$ receptor-chloride channel complex. The initial stage was to characterize the mechanism of action of dieldrin on the whole-cell GABA-induced chloride current in rat DRG neurons in primary culture. The second stage was to identify the GABA receptor subunits and their combinations that were required for the dieldrin action using human embryonic kidney (HEK 293) cell lines in which GABA receptor subunits had been expressed in various combinations.

The $GABA_A$ receptor-chloride channel complex is known to be the target site of dieldrin and lindane. In order to elucidate the mechanism of dieldrin interaction with the GABA system, whole-cell patch clamp experiments were performed with rat DRG neurons in primary culture [15]. When co-applied with GABA, dieldrin exerted a dual effect on the GABA-induced chloride current (Fig. 2). The chloride current induced by 10 μM GABA was greatly enhanced by the first 20-s co-application with 1 μM diel-

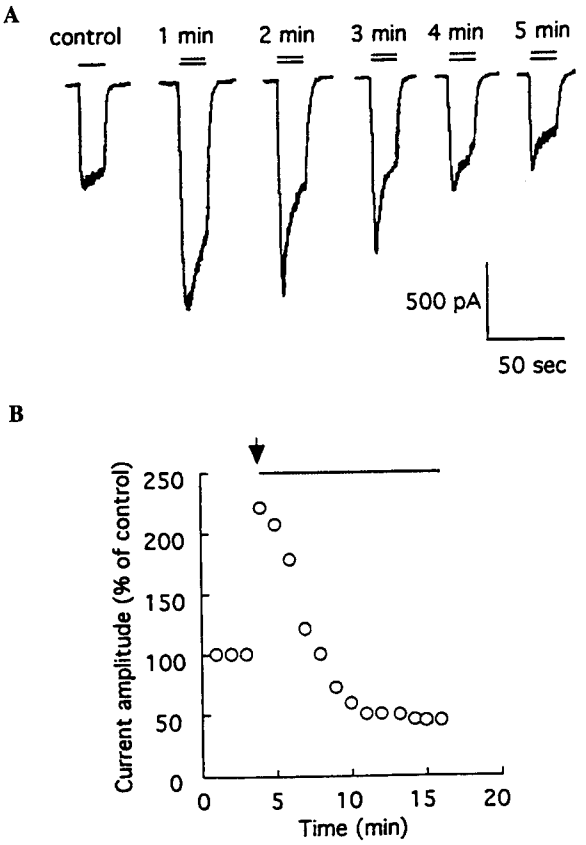


Fig. 2. Effects of dieldrin on GABA-induced chloride currents in a rat DRG neuron. (A) Current records in response to 20-s application of 10 μM GABA (solid bar) and to co-application of 10 μM GABA and 1 μM dieldrin (dotted bar) at the time indicated after taking control record. The peak amplitude of current is greatly enhanced but gradually decreases during repeated co-applications. Desensitization of current is accelerated. (B) Time course of the changes in peak current amplitude before and during repeated co-applications (dotted line). (Reproduced with permission from Ref. [15]. Copyright 1994 by the American Society for Pharmacology and Experimental Therapeutics).

drin, but the enhancement subsided during repeated co-applications, and the current was eventually suppressed below the control level. No recovery occurred after a prolonged washing with dieldrin-free solution. Desensitization of the chloride current was accelerated by dieldrin. However, when the period of co-application was limited to 2 s, which was short enough to avoid desensitization, no suppression of current was observed during repeated co-applications and recovery occurred after washing. The desensitization and suppression occurred with an EC_{50} of 92 nM, whereas the enhancement required a higher EC_{50} at 754 nM. The GABA-induced chloride current comprised 2 components, one with a high sensitivity to dieldrin suppression with an EC_{50} of 5 nM and the other with a lower sensitivity with an EC_{50} of 92 nM. Dieldrin exerted its inhibitory effect on the GABA-induced current regardless of the presence or absence of pentobarbital and chlordiazepoxide. However, its effect was attenuated by the presence of picrotoxin. Furthermore, dieldrin suppressed the GABA-induced chloride current in noncompetitive manner. These results indicate that dieldrin binds to the picrotoxin site which is closely associated with the chloride channel. The suppressive action of dieldrin on the GABA-induced chloride current is deemed directly responsible for excitatory systems of poisoning in animals, but the role of the enhancing action is not clear.

2.2. GABA receptor subunits required for dieldrin action

Molecular biological studies have recently revealed the structures of several subunit proteins of the $GABA_A$ receptor-chloride channel complex. The complex is believed to be a pentameric protein comprising 5 subunits in various combinations [16]. At present 6 αs, 4 βs, 3 γs, 1 δ and 2 ρs subunit are known to exist [17,18]. Different subunits have been shown to differ considerably in their sensitivity to the actions of various drugs [19]. The presence or absence of the $\gamma 2$ subunit in the $GABA_A$ receptor structure influences the action of benzodiazepines and Zn. However, the subunit requirements for the action of barbiturates, TBPS and picrotoxin on the $GABA_A$ receptor-channel complex are controversial. Nothing is known about the role of the GABA receptor subunits in the action of cyclodiene and lindane-type insecticides. The observed dual action of dieldrin and the high and low sensitivity to dieldrin may be due to its selective action on different combinations of subunits.

We have studied the differential effects of dieldrin on the GABA-induced chloride current of human embryonic kidney cells expressing 3 different combinations of α, β and γ subunits [20]. The EC_{50} values for GABA induction of current were estimated to be 9.8 μM for the $\alpha 1\beta 2\gamma 2$s combination, 2.0 μM for the $\alpha 1\beta 2$ combination and 3.0 μM for the $\alpha 6\beta 2\gamma 2$s combination. When co-applied with GABA, dieldrin exerted a dual effect, enhancement and suppression, on the GABA-induced chloride currents in the $\alpha 1\beta 2\gamma 2$s and $\alpha 6\beta 2\gamma 2$s combinations. However, only suppression was observed in the $\alpha 1\beta 2$ combination, indicating that the γ subunit is necessary for dieldrin's enhancing effect. Dieldrin was more efficacious in enhancing the current in the $\alpha 6\beta 2\gamma 2$s combination than in the $\alpha 1\beta 2\gamma 2$s combination, indicating some specific role of α subunits in the dieldrin enhancement of current. Dieldrin suppressed the GABA-induced current in a non-competitive manner, with an EC_{50} value of 2.1 μM for $\alpha 1\beta 2\gamma 2$s, 2.8 μM for $\alpha 1\beta 2$ and 1.0 μM for $\alpha 6\beta 2\gamma 2$s combination. These results indicated that dieldrin suppression did not require specific subunit combinations among the 3 tested.

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Toxicology Letters

Towards the development of ryanoid insecticides with low mammalian toxicity

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Abstract

The effects of ryanodine and 9,21-didehydroryanodol on mammalian and insect muscles have been compared. Whereas ryanodine markedly affected the functioning of insect and mammalian muscles, the action of 9,21-didehydroryanodol was mainly restricted to the insect muscles. Unlike ryanodine, 9,21-didehydroryanodol did not elicit a contracture from insect muscles, yet it inhibited muscle contractions. This inhibition is associated with changes in the ion selectivities of potassium channels located in the muscle plasma membrane. These changes lead to a decline in muscle resting potential and loss of excitability.

Keywords: Ryanodine; 9,21-Didehydroryanodol; Mouse diaphragm muscle; Insect muscle; Potassium channels

1. Introduction

Extracts from the ground stem-wood of the plant *Ryania speciosa* have been used as insecticides for about 50 years. A major component of these extracts is the alkaloid ryanodine, a compound that has been used extensively to study excitation-contraction coupling of muscle. Ryanodine (ryanodyl 3-(pyrrole-2-carboxylate)) is a complex bridged diterpene heptol (Fig. 1) [1]. It is a selective insecticide, but one with high mammalian toxicity (Table 1). Ryanodine receptors with high binding affinities for the alkaloid (Table 2) have been isolated from a variety of muscles of vertebrates and invertebrates, and some of these receptors have been cloned. They are located in the region of the cisternae of muscle sarcoplasmic reticulum (SR) where they influence release of Ca^{2+} from SR.

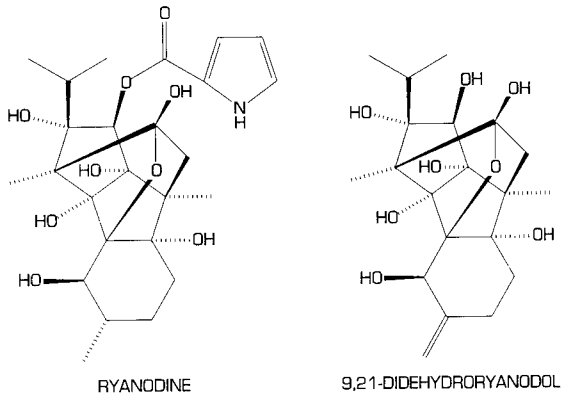


Fig. 1. Comparison of the structures of ryanodine and 9,21-didehydroryanodol.

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Table 1
Ryanoids – mouse and insect toxicology (LD_{50}; $\mu g\ g^{-1}$; 24 h)

	Ryanodine	9,21-Didehydroryanodol
Mouse (i.p.) (1)	0.1	>20
House Fly (i.t.)[a] (1)	0.09	0.36
Cockroach (adult) (i.t.)[a]	0.8	9 (17[b])
Locust (nymph) (i.t.)[a]	0.7	~5 (5[b])

The insect data are more accurately 50% knockdown values rather than LD_{50} values (see text); i.p., intraperitoneal injection; i.t., intrathoracic injection.
[a]Plus piperonyl butoxide (1–7 $\mu g\ g^{-1}$).
[b]Topical application.
(1) Jefferies, P.R. et al. (1987) J. Med. Chem. 30, 710.

Table 2
Ryanoids – Action on skeletal muscle ryanodine receptors (IC_{50}; nM)

	Ryanodine	Ryanodol
Rabbit	10 (13[a])	3500 (1000[b])
Mouse	2.6	901
House Fly	2.8	395
Cockroach	3.0	379

After 80- to 120-min incubation with muscle protein at 37°C.
[a] Value for 9,21-didehydroryanodine.
[b] Value for 9,21-didehydroryanodol.
From Waterhouse et al. [2] and Lehmberg and Casida [4].

This influence is thought to account for failure of excitation-contraction coupling and for the appearance of a muscle contracture during ryanodine poisoning. Studies of [^{3}H]ryanodine binding to mammalian muscle (Table 2) suggest that SR ryanodine receptors play an essential role in the mammalian toxicity of ryanoids. Also, the IC_{50} value for binding of [^{3}H]ryanodine to insect SR ryanodine receptors (Table 2) is in the range that would be expected if these receptors play a pivotal role in ryanodine toxicity.

In insects and mammals ryanodine can cause muscle rigor, although the final outcome of ryanodine poisoning may be either a tonic or a flaccid paralysis. When ryanodine enters an insect it is rapidly metabolised, probably by oxidation. As a result, an insect that suffers knockdown by this alkaloid may eventually recover. To prevent this, piperonyl butoxide may be used as a cytochrome P_{450}-oxidase inhibitor [1], in which case the insect toxicity of ryanodine is sometimes irreversible. Casida and colleagues compared the toxicities of ryanodine and analogues to insects and mammals (Table 1) [1–4]. The non-selective toxicity of ryanodine was confirmed, but two alcohols, ryanodol and 9,21-didehydroryanodol (Fig. 1), exhibited a high selectivity for insects over mammals (Table 1). In this study, the actions of ryanodine (Fig. 1) and 9,21-didehydroryanodol (Fig. 2) (ryanodol was not available, but studies by Casida and colleagues have shown that its pharmacological properties are very similar to those of 9,21-didehydroryanodol (Table 2)) on insect and mammalian muscles are compared in an effort to establish the reasons for the insect selectivity of the alcohol. If 9,21-didehydroryanodol paralyses insects by interacting with a site that is distinct from the SR ryanodine receptor and if this site is not present in mammalian muscle, then we might have an explanation for the insect selectivity of this compound. Is there any evidence to support this proposal? Ryanodol has a low affinity for insect and mammalian SR ryanodine receptors, so if the insect toxicity of this ryanoid results from muscle paralysis then presumably another site must be targeted. What is this site? It was discovered over three decades ago that the action of ryanodine on locust leg muscle is not restricted to the excitation-contraction coupling system, but also involves the electrically-excitability of this muscle [5]. The effects of ryanodine on the contractile system of locust muscle are preceded by, and then accompanied by, changes in electrical excitability, with the appearance of all-or-none action potentials in a system that normally exhibits graded electrical

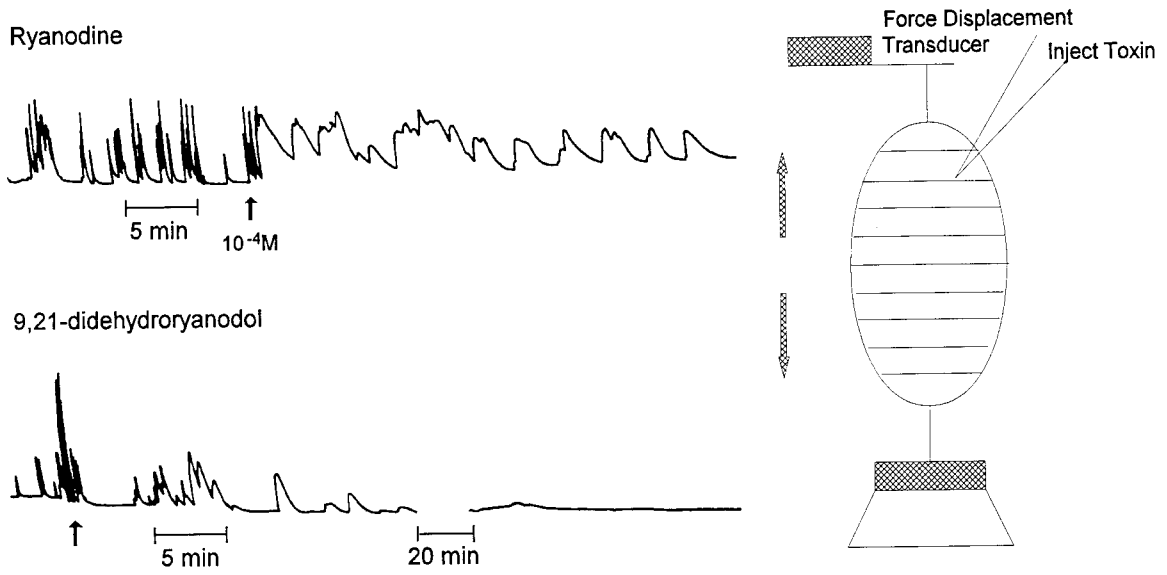


Fig. 2. Effects of 10^{-4} M ryanodine and 10^{-4} M 9,21-didehydroryanodol on contractions of intact, 'wandering' blowfly (*Lucilia sericata*) larvae. The experimental arrangement is illustrated on the right. The insect was attached by crocodile clips (not shown) to a force displacement transducer (strain gauge) which measured the body contractions of the insect. The later occurred in bursts at ~1-min intervals. Following injection of 1 μl of 10^{-4} M ryanodine through a microsyringe the body exhibited a contracture, and although rhythmic contractions continued for some time they were eventually lost. The final outcome of ryanodine poisoning was a tonic paralysis (not shown). Injection of 1 μl of 10^{-4} M 9,21-didehydroryanodol did not cause a contracture, but the rhythmic contractions were eventually lost to produce a flaccid paralysis.

excitability. Usherwood [5] proposed that the changes in excitability might involve the K^+ conductance properties of the plasma membrane. Do ryanodol and 9,21-didehydroryanodol also affect the electrical excitability of insect muscle and could this account for their toxicity to insects? This membrane of locust skeletal muscle contains two types of K^+ channel: a maxi, Ca^{2+}-activated, 170pS channel; and an inward rectifier of 35pS conductance, the gating kinetics of which are not influenced by Ca^{2+} [6]. Single channel studies undertaken on patches of locust muscle membrane have confirmed that ryanodine influences these channels, but in an unexpected fashion [7–9]. The alkaloid converts the K^+ channels to channels that no longer discriminate well between K^+ and Na^+, a change that has inevitable consequences for the electrical properties of the plasma membrane, for the mechanical responsiveness of muscle and for muscle homeostasis. If 9,21-didehydroryanodol has a similar action on the K^+ channels of insect muscle but not those of mammalian muscle, could this account for the insect selective toxicity of this ryanoid, bearing in mind that 9,21-didehydroryanodol has a low affinity for ryanodine receptors?

2. Results

2.1. Toxicological studies

These studies were undertaken on adult cockroaches (*Periplaneta americana*), 3rd instar locusts (*Schistocerca gregaria*) (Table 1) and 'wandering' larvae of blowfly (*Lucilia sericata*) (data not shown). Ryanodine or 9,21-didehydroryanodol was applied topically, with and without piperonyl butoxide, and by injection, with and without piperonyl butoxide. The results of these studies were similar to those reported for house flies (Table 1) and cockroaches [1,2].

2.2. Injection of ryanoids into blowfly larvae

When 'wandering' blowfly (*L. sericata*) larvae are injected with 1 μl of 10^{-4} M ryanodine, they are rapidly paralysed, but after 24–48 h they recover. Recovery is much slower if larvae are exposed to piperonyl butoxide before ryanodine injection. The paralysis by ryanodine is accompanied by contracture of the bodywall musculature. In contrast, 1 μl of 10^{-4} M 9,21-didehydroryanodol causes a flaccid paralysis of slower onset. Recovery is again evident after 24–48 h, but can be slowed by prior treatment of larvae with piperonyl butoxide. To further investigate the paralyses caused by the two ryanoids, small crocodile clips were attached to each end of a larva, one end was fixed and the other was attached to a strain gauge (Fig. 2). After recording the bodywall contractions for about 1 h, either 1 μl 10^{-4} M ryanodine in saline or 1 μl 10^{-4} M 9,21-didehydroryanodol in saline or 1 μl saline alone was injected into the larva. The latter sometimes caused a brief increase in the frequency of bodywall contractions, but was otherwise inactive. Ryanodine almost immediately caused a body shortening, due to contracture of longitudinal bodywall muscles, that did not decline in magnitude for the rest of the recording (often >3 h). Also, the spontaneous bodywall muscle contractions that are normally recorded from these preparations in the absence of ryanoid gradually decreased in frequency. 9,21-Didehydroryanodol inhibited the bodywall muscle contractions but did not elicit a contracture. The different actions of ryanodine and 9,21-didehydroryanodol on insect muscle were further established when these compounds were applied to either retractor unguis [10] or extensor tibiae (Fig. 3A) [5] muscles isolated from metathoracic legs of adult locusts (*S. gregaria*). Ryanodine, but not 9,21-didehydroryanodol, elicited contractures (Fig. 3B), although both ryanoids reduced the mechanical responsiveness of these muscles.

2.3. Effects of ryanoids on locust muscle

The neurally-evoked twitch contractions of locust retractor unguis muscle are inhibited by

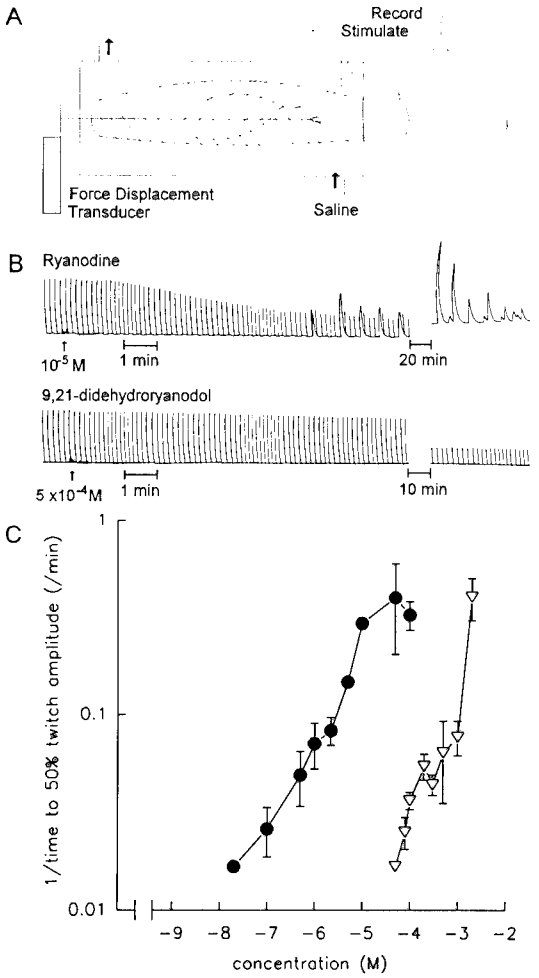


Fig. 3. Effects of ryanoids on the neurally-evoked mechanical responses of locust (*Schistocerca gregaria*) metathoracic extensor tibiae muscle. The experimental set-up, that also enables concomitant recordings of muscle fibre membrane potentials, is illustrated in A. The top recording in B shows the effects of bath-applied ryanodine (10^{-5} M) on the neurally-evoked twitch contractions of the extensor tibiae muscle. Note the initial appearance of prolonged twitches followed by the development of a contracture. The responses to neural stimulation eventually failed. The effects of 5 × 10^{-4} M 9,21-didehydroryanodol are illustrated in the bottom recording B. Note that although the concentration of this ryanoid was much higher it did not elicit a muscle contracture, although it did reduce the amplitude of the twitch contraction. The effects of ryanodine (●) and 9,21-didehydroryanodol (▽) on the amplitude of the neurally-evoked twitch contraction of locust metathoracic retractor unguis muscle are shown in C. The time taken by these ryanoids to reduce the twitch contraction amplitude by 50% is plotted as a reciprocal against ryanoid concentration. Note that according to this measure ryanodine is more potent than the alcohol (but see text).

ryanodine and 9,21-didehydroryanodol. However, there are significant quantitative differences in the potencies of these compounds. In Fig. 3C the reciprocal of the time taken to inhibit the twitch by 50% is plotted against log ryanoid concentration. It is clear from the plots that, according to this measure, ryanodine is more potent than 9,21-didehydroryanodol. This is seemingly a surprising result in view of the similar insect toxicities of these compounds? However, the relatively short lifetime of the muscle preparations (max. 12 h), makes them unsuitable for studying slow changes in mechanical responsiveness induced by compounds such as 9,21-didehydroryanodol. In the toxicological studies on whole insects, KD_{50} (50% knockdown) values for ryanodine and 9,21-didehydroryanodol could not be accurately determined until ~48 h after application of the ryanoids.

2.4. Effects of ryanoids on mammalian muscle

Diaphragm muscles, with their associated phrenic nerves, were dissected from adult mice, attached at one end to the base of a bath and at the other end to a strain gauge and perfused with O_2/CO_2 aerated saline at 37°C. Application of $>10^{-9}$ M ryanodine caused a muscle contracture and reduction in twitch contraction (i.e. the mechanical response to stimulation of the phrenic nerve). In contrast, 9,21-didehydroryanodol, at concentrations as high as 10^{-4} M, did not elicit a contracture and had little effect on the amplitude of the twitch (Fig. 4A,B). Higher concentrations of the alcohol sometimes caused a slight contracture, and sometimes significantly reduced the twitch contraction amplitude (data not shown). However, in comparison with ryanodine, the lack of action of 9,21-didehydroryanodol on mouse diaphragm muscle was quite remarkable, but it was not unexpected in view of the toxicity studies described above. The difference between the two compounds is graphically illustrated in Fig. 4B. Even when 9,21-didehydroryanodol was applied for long periods, it had little effect on the amplitude of the muscle twitch contraction.

2.5. Changes in muscle resting potential

The appearance of muscle contractures elicited by ryanodine give added weight to the view that the insect toxicity of this compound involves its

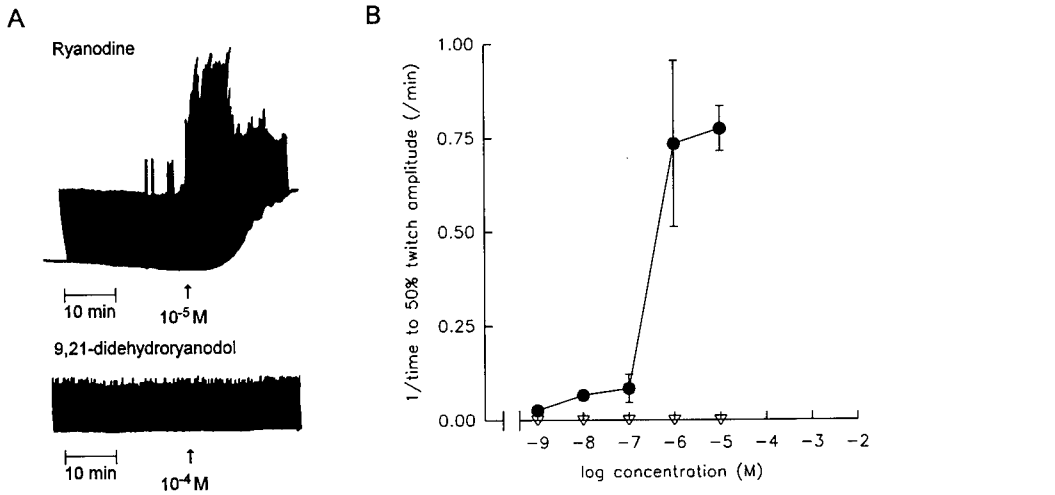


Fig. 4. Effects of ryanoids on the neurally-evoked twitch contractions of mouse diaphragm muscle. In A, 10^{-5} M ryanodine causes a large contracture and modifies the twitch contraction (top trace), whereas a 10-fold higher concentration of 9,21-didehydroryanodol did not cause a contracture and had no effect on the twitch contraction (bottom trace). In B, the times taken for the twitch contraction amplitude to decline by 50% in the presence of ryanodine (●) and 9,21-didehydroryanodol (▽) are plotted as reciprocals against ryanoid concentration.

interaction with SR ryanodine receptors. What then accounts for the paralysis caused by 9,21-didehydroryanodol? Is this due to alterations in the electrical excitability of muscle as proposed by Usherwood [5] in 1962? Like ryanodine, 9,21-didehydroryanodol converts the graded electrical excitability of locust muscle to all-or-none excitability, but this change would not account for loss of contractile function. However, Usherwood [5] noted that during long-term exposure of locust muscle to ryanodine the resting potential (E_m) of the muscle declines, a change that could eventually lead to a loss of mechanical responsiveness. The resting tension of locust (*S. gregaria*) metathoracic extensor tibiae muscle has been recorded concomitantly with E_m before and during application of the two ryanoids (Fig. 3A). Over 1- to 3-h periods when a dissected muscle is exposed to toxin-free locust saline there is no change in resting tension and E_m (data obtained by sampling the membrane potentials of 5–10 muscle fibres each 20 min using intracellular micropipettes filled with 3 M KCl) remains constant at approx. −60 mV. At concentrations lower than 10^{-4} M, ryanodine slowly reduces E_m, but any changes in this parameter occur after the appearance of a muscle contracture. With 10^{-4} M ryanodine, the change in E_m occurs before muscle contracture ensues. 9,21-Didehydroryanodol also lowers E_m, the rate of change in E_m being directly proportional to the ryanoid concentration. However, the changes in E_m are never accompanied by the appearance of a muscle contracture (Fig. 3B). With both ryanoids there is sometimes a slight increase in E_m almost immediately after their application [5]. What is the basis for the changes in E_m observed with the two ryanoids? The single channel studies summarised below provide a possible explanation.

2.6. Single channel studies

Patches were made from the plasma membrane of metathoracic extensor tibiae fibres of adult locusts (*S. gregaria*). Cell-attached, inside-out and outside-out patches were used [7–9]. Patches were also made from plasma membrane of mouse (30- to 45-day-old) interosseal muscles dissected from hind feet. Ryanodine had similar effects on the two types of K^+ channel present in patches of locust muscle membrane. At concentrations as low as 10^{-9} M, ryanodine irreversibly shifts the reversal potential (V_{rev}) from about −60 mV towards zero; with 10^{-6} M ryanodine, V_{rev} is reduced to about −20 mV. This action of ryanodine has an apparent dissociation constant (K_d) of 28 nM. Although the magnitude of the change in V_{rev} induced by 9,21-didehydroryanodol is less than that for ryanodine, the K_d is lower, i.e. 1.5 nM. The effect of the ryanoids on V_{rev} was independent of the Ca^{2+} concentration on either side of a membrane patch. Neither ryanodine nor 9,21-didehydroryanodol affected the conductances of the K^+ channels and their kinetics, although the latter have not yet been studied systematically during ryanoid application. Ryanodine and 9,21-didehydroryanodol also changed V_{rev} of a Ca^{2+}-activated channel in the plasma membrane of mouse skeletal muscle. Although the K_d values were similar to those for the locust, maximal changes in V_{rev} were lower than for locust muscle (Table 3).

Table 3
Ryanoid induced changes in permeability of mouse muscle potassium channel (BK channel) and locust-muscle potassium channels. V_{rev}, reversal potential of potassium channel in the presence of ryanoid. $V_{rev}(0)$, reversal potential before ryanoid application K_d, apparent dissociation constant

	$(V_{rev}(0) - V_{rev})/V_{rev}(0)$	Kd_{50} (nM)
Mouse		
Ryanodine	0.33	2.1 ± 0.7
9,21-Didehydroryanodol	0.18	8.7 ± 1.9
Locust		
Ryanodine	>0.8	28 ± 15
9,21-Didehydroryanodol	0.42	1.5 ± 0.1

3. Discussion

The changes in membrane excitability observed during application of ryanodine and 9,21-didehydroryanodol to locust skeletal muscle

could be accounted for by the effects of these compounds on the ion selectivities of K^+ channels of the plasma membrane. Ba^{2+} and some other divalent cations also convert the graded responsiveness of insect muscle to all-or-none responsiveness, but by blocking plasma membrane K^+ channels rather than by changing their ion selectivities [1]. Is there any evidence that the Ca^{2+} released from intracellular stores by ryanodine is responsible for the changes in properties of the K^+ channels during ryanodine application to locust muscle? This seems unlikely because these changes were also observed during application of ryanodine to excised patches of muscle plasma membrane. Also, it has been established that 9,21-didehydroryanodol has a low affinity for SR ryanodine receptors and, therefore, does not seemingly release Ca^{2+} from intracellular stores of locust muscle, yet this compound alters the ion selectivities of the K^+ channels in this tissue. The changes in K^+ channel ion selectivity occur very quickly after application of 10^{-4} M ryanoid, as does the appearance of all-or-none action potentials, yet the contractile system is unaffected at this time. However, twitch contractions may be enhanced and repetitive contractions of variable amplitude may follow a single brief stimulus. With 10^{-4} M ryanodine, a muscle contracture soon ensues, E_m declines and the electrical excitability of the muscle falls. The decline in E_m and electrical excitability are probably causally related phenomena, but is the fall in E_m associated with a rise in intracellular free Ca^{2+}? Neither of the K^+ channels inactivate during ryanodine treatment. Although the open probability of the 170pS channel is increased when intracellular free Ca^{2+} is raised, this influence of Ca^{2+} saturates when the Ca^{2+} concentration reaches 10^{-9} M. The release of Ca^{2+} from intracellular stores during ryanodine poisoning is expected to produce much higher concentrations than this. It is possible of course, that release of Ca^{2+} from intracellular stores during ryanodine treatment perturbs other components of the system that controls E_m. However, 9,21-didehydroryanodol does not seemingly raise the intracellular free Ca^{2+} concentration, yet it does lower E_m of locust muscle. The change in ion selectivities of the plasma membrane K^+ channels would lead to a fall in E_m if the plasma membrane Na^+/K^+ pump could not cope with the resultant influx of Na^+ ions. In intact insects, oxygen consumption increases by ~20% after injection with *Ryania speciosa* extract [12]. Such a change would be consistent with an increase in Na^+/K^+ ATPase activity. During application of 10^{-4} M ryanoid the fall in E_m is initially rapid, but then it declines more slowly. In fact, the high Cl^- conductance of locust muscle [13] will slow down the rate of change of E_m.

Does the action of 9,21-didehydroryanodol on the K^+ channels of locust muscle and its low affinity for SR ryanodine receptors in mammals account for the insect selective toxicity of this ryanoid as suggested in Fig. 5? Undoubtedly, the low affinity of 9,21-didehydroryanodol for mam-

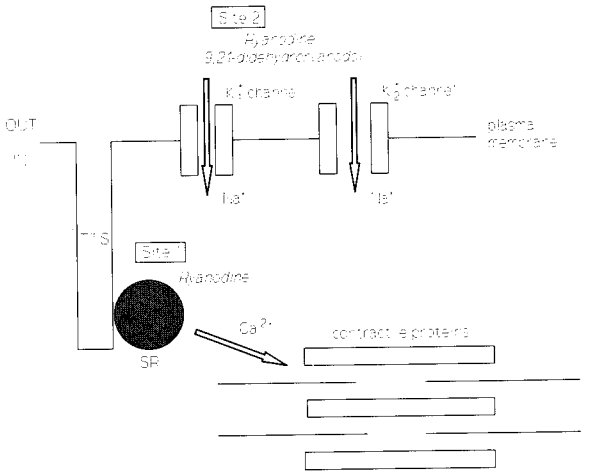


Fig. 5. Diagrammatic representation of ryanoid action on an insect muscle fibre. Two sites of action have been identified. Site 1 is the SR ryanodine receptor. When ryanodine binds to this receptor the free intracellular Ca^{2+} concentration of the muscle fibre is raised, excitation-contraction coupling fails and a contracture is elicited. Ryanodine has a high affinity for this site; the affinity of 9,21-didehydroryanodol is much lower. Site 2 involves two types of K^+ channel located in the plasma membrane of the muscle fibre. Ryanodine and 9,21-didehydroryanodol have high affinities for this site. Conversion of the K^+ channels to channels that discriminate poorly between K^+ and Na^+ leads to an increase in the intracellular Na^+ concentration of the muscle, a decline in the potential difference across the plasma membrane and loss of electrical and mechanical excitabilities.

malian SR ryanodine receptors and its weak affect on plasma membrane K^+ channels in these animals at least contributes to its low mammalian toxicity. The influence of 9,21-didehydroryanodol on insect muscle K^+ channels could account for the flaccid paralysis that is seen during its topical application to insects, but further studies will be required to establish whether this is the only reason for its insect toxicity. Also, the action of 9,21-didehydroryanodol on plasma membrane of insect muscle may not be restricted to K^+ channels. Studies currently in progress are designed to see whether other voltage-gated channels are involved. Is 9,21-didehydroryanodol a possible insecticide? In view of its low mammalian toxicity and its relative high insect toxicity the answer must be in the affirmative. Unfortunately, this ryanoid is a natural product which is costly to extract and which would be difficult and expensive to synthesise. However, it may be possible through structure/activity studies, allied to a programme of synthetic chemistry, to design ryanoids that are simpler structurally than the natural product and that have even greater insect selectivity. Nevertheless, it may be wise to inject a note of caution here. Although 9,21-didehydroryanodol has only a slight effect on ion selectivity of a K^+ channel in the plasma membrane of mouse muscle and this does perturb muscle function, at least in short-term studies, this mammalian toxicity should not be ignored.

Acknowledgements

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Toxicology Letters

Calcium channels as target sites of heavy metals

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Abstract

Zinc (Zn), aluminium (Al), mercury (Hg), methylmercury (MeHg) and lead (Pb) extracellulary applied reduce voltage-activated calcium channel currents (VACCCs); Pb and Al also reduce *N*-methyl-D-aspartate (NMDA)-activated channel currents (NACCs). Pb is most effective in reducing VACCCs, with an IC_{50} of 0.46 μM, followed by Hg ($IC_{50} = 1.1$ μM) and MeHg ($IC_{50} = 2.6$ μM). Zn and Al were less potent ($IC_{50} = 69$ and 84 μM, respectively). Al acts on channels in the open state; its effect is pH dependent. The effects of Pb were specific for VACCCs and NACCs. Hg, Al and Zn had only minor effects on voltage-activated potassium and sodium channels, while MeHg reduced potassium channel currents ($IC_{50} = 2.2$ μM) and, at higher concentrations, sodium channel currents ($IC_{50} = 12.3$ μM). Al also reduced other receptor-activated channel currents. These results demonstrate that a variety of metal species produce different actions at the level of the cell membrane.

Keywords: Voltage-activated calcium channel currents (VACCCs); NMDA-activated channel currents (NACCs); Lead (Pb); Mercury (Hg); Methylmercury (MeHg); Aluminum (Al); Zinc (Zn)

1. Introduction

Learning and memory processes are triggered by a rise of the intracellular calcium concentration. While the extracellular calcium concentration is in the millimolar range, the intracellular calcium concentration in neurons is very low (10^{-7}–10^{-6} M) and closely regulated. Extracellular calcium enters neurons through calcium permeable 'gates'. These calcium permeable channels are opened by 2 entirely different mechanisms: the first is the receptor-activated type, opened by the agonist *N*-methyl-D-aspartate (NMDA), while the second type is opened by depolarization of the membrane potential. Hence, the first type is named the NMDA-activated channel (NAC) and the second the voltage-activated channel (VACC). NACs have a magnesium and a zinc binding site and are modulated by different metals [1]. For VACCCs several subtypes have been described [2], all of them are highly selective for calcium.

Zinc (Zn) is an essential metal, while other metals and metal compounds such as mercury (Hg), methylmercury (MeHg), aluminum (Al) and lead (Pb) are toxic and interfere with cognitive functions. We examined the effects of these metals using the whole-cell patch clamp technique with either cultured rat dorsal root ganglion (DRG) neurons for recording voltage-activated calcium channel currents (VACCCs), or

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acutely isolated rat hippocampal neurons for NMDA-activated channel currents (NACCs).

2. Materials and methods

Cultures of neurons from the DRG of young rats were used to study the actions of metals on VACCCs. Since DRG neurons do not express NMDA receptors, acutely isolated hippocampal neurons were used to study NACCs.

2.1. Preparation and recordings of hippocampal neurons

Hippocampal slices from 2–3-week-old rats were prepared and incubated for 40 min in Kreb's Ringer solution (Table 1; bubbled with carbogen). The slices were transferred to a low calcium medium (Table 1) for 10 min and treated with *Aspergillus oryzae* protease (Sigma; 3.5 units/mg). These slices could be maintained for up to 10 h at room temperature.

The neurons were isolated by mechanical teasing of the slices using a pair of glass needles. In most cases CA1 neurons were used for recording. For whole cell patch-clamp recordings the solution was changed to the recording solution as indicated in Table 1.

2.2. Preparation and recordings of DRG neurons

DRG were removed from 2–4-day-old rat pups and stored in phosphate-buffered saline. The ganglia were incubated in 0.9 ml F14 medium (DUN, Germany) containing 10% horse serum (GIBCO, USA) and 0.1 ml collagenase (12.5 mg/ml; Sigma Type II), for 13 min at 37°C. After the incubation the collagenase was removed and the ganglia were washed in F14 medium 3–5 times. Trypsin stock (0.1 ml, from 25 mg/ml; Sigma Type IX) and 0.9 ml F14 were added and the ganglia were incubated for 6 min at 37°C. After removing the trypsin-containing solution, the ganglia were washed with F14 and transferred to a plastic test tube containing 2 ml F14 medium with 10% horse serum, and DNAase (1 mg/10 ml; Sigma Type II).

Table 1
Ionic composition of external and internal solutions

	Tyrodes	Ca-currents		Enzyme-Solution	Asp/Gly		
		External	Internal		External		Internal
					Krebs-Ringer	Asp/Gly	
NaCl	145.0			125.0	125.0	140.0	
KCl	2.5			3.7	3.7	5.0	
HEPES	10.0	10.0	10.0	5.0	5.0	10.0	
Glucose	10.0	10.0		10.0	10.0	10.0	
$CaCl_2$	1.5			1.0	1.8	1.8	
$MgCl_2$	1.2	1.0	4.0	0.5	1.3		
TEA-Cl		130.0					
$BaCl_2$		10.0					
TTX		0.0004					
CsCl			140.0				
EGTA			10.0	1.0			
Na-ATP[1]			2.0				
KH_2PO_4				1.2	1.2		
$NaHCO_3$				25.0	25.0		
KF							100.0
Tris–Cl							30.0
pH	7.4	7.2	7.2	7.4	7.4	7.3	7.2

All concentrations are given in mM.
[1] To avoid rundown of the calcium channel current due to a reduction of the energy pool.

The ganglia were triturated with a fire-polished Pasteur pipette until they were dispersed and the medium appeared opaque. The debris was removed by filtering through a nylon mesh (ϕ200 μm). The cell suspension (50–100 μl) was placed in small petri dishes (Falcon, 'easygrip') and incubated for 2 h at 37°C with 5% CO_2. Then the dishes were filled with 1 ml F14 containing 10% horse serum. The neurons were used within 3 days after preparation.

2.3. Recording technique and analysis

The neurons were patch-clamped in the whole cell configuration using a HEKA EPC-9 patch-clamp amplifier controlled by a computer. Electrode resistance was between 2 and 5 MΩ. The compositions of the internal and external solutions (Tyrode and calcium current solutions) are shown in Table 1. All experiments were conducted at room temperature.

Cells were clamped at −80 mV (DRG neurons) or −60 mV (hippocampal neurons). All solutions used for recordings are shown in Table 1.

The dose-response relations for the effects of the various metals on VACCCs and NACCs of the neurons were determined by fitting mean currents to the equation:

$$I_{Ca^{2+}(X)} = I_{Ca^{2+}(control)}(1/1 + (K_m/X^n)$$

where $I_{Ca^{2+}(X)}$ is the calcium current measured in the presence of a given concentration of a metal, $I_{Ca^{2+}(control)}$ is the calcium current without the metal, K_m is the apparent dissociation constant, and n is the Hill coefficient.

Metals were added in different concentrations to the external solution immediately before application to the neurons.

3. Results

3.1. Actions of Pb and Al on NACCs

Both Pb and Al reduce currents activated through the NMDA receptor. Application of the agonists aspartate (500 μM) or NMDA (1 mM) simultaneously with glycine (20 μM) resulted in an inward current. Application of Pb or Al together with the agonist results in a partially reversible, dose-dependent reduction of the current through the channel/receptor complex [3–5]. The current was reduced to half at Pb concentrations between 20 and 50 μM, and at Al concentrations of less than 50 μM (i. e. 1.4 μg/ml Al). Both metals reduced the receptor-activated currents over the whole voltage range without changing the reversal potential; no voltage dependence was found. Preincubation with Pb increased the reduction of the receptor-activated current [5]. We did not test the action of Al with preincubation, but we have shown that it reduces glutamate and AMPA-activated currents in a similar concentration range [4]. As Alkondon et al. [6] have proven, the effect of Pb is specific for NMDA-activated currents. Only minor actions on kainate- or quisqualate-activated currents, with the same concentrations of Pb which block NMDA-activated currents, have been shown. No interactions of Pb with the metal binding sites of Zn or Mg of the NMDA-receptor/channel complex have been reported [7].

3.2. Actions of Pb, Al, Hg, MeHg and Zn on VACCCs

3.2.1. Actions on calcium channel subtypes

3.2.1.1. Actions on L-/N-type channels. Depolarizing from the holding membrane potential of −80 mV to 0 mV for 75 ms results in activation of high VACCCs. (We have not tested for P-type calcium channel currents).

All metals tested (Pb, Al, Hg, MeHg and Zn) reduced the peak of VACCCs. Pb was most effective in reducing these currents with an IC_{50} of 0.46 μM [3], followed by Hg ($IC_{50} = 1.1$) [8], MeHg ($IC_{50} = 2.6$ μM) [9], Zn ($IC_{50} = 69$ μM) [10] and Al ($IC_{50} = 83$ μM) [11]. The Hill coefficient was close to 1 for Pb, Hg, MeHg and Zn. When Al was applied the Hill coefficent varied with pH, from 2.8 (pH 7.7) to 2.2 (pH 7.3) to nearly 1.4 (pH 6.7) [12].

While the reduction of the calcium channel current by Pb was reversible up to 60%, the

actions of Al and Zn were less reversible (up to 30%). The blocking effect of Hg or MeHg was not more than 10% reversible.

3.2.1.2. Actions on T-type calcium channel currents. In about 5% of the neurons tested low VACCCs (through the voltage-activated T-type calcium channels) could be found. These currents were activated maximally by a voltage step to −30 mV. Pb, Hg, MeHg and Al reduced T-channel currents in a similar concentration range as currents through high VACCCs. Zn was different: a concentration of this metal which reduced the peak current through the L-/N-type channels by less than 10% blocked the current through the low VACCs almost completely (>80%) [13]. Due to the low number of recordings with T-type channel currents, we were not able to determine a concentration response relationship.

3.2.2. Specificity

We tested the actions of Pb, Al, MeHg and Zn on voltage-activated sodium and potassium channels. (We have not tested the specificity of Hg). All metals reduced these currents to some degree, but Pb, Al or Zn had only a slight action (<10% reduction) on voltage-activated potassium or sodium channels at concentrations that reduce the currents through VACCs by more than 80% [14]. MeHg was about as effective in reducing the currents through potassium channels ($IC_{50} = 2.6\ \mu M$) as it was in VACCCs, while sodium channel currents were less sensitive to MeHg ($IC_{50} = 12\ \mu M$) [15].

3.2.3. Time course and use dependence

After application of Pb, Hg, MeHg or Zn, a new and lower steady state was reached within a few minutes [14,15]. With Al the time to reach a steady state was about twice as long [11].

The actions of Pb and Zn on VACCCs did not depend on an open channel state. With Hg or MeHg the VACCCs were partly reduced when the channels were activated. In the presence of Al the VACCCs were not reduced when the protocol of channel activation was resumed after several minutes (up to 6 min) [11].

3.2.4. Current-voltage relation

All metals tested reduced the VACCCs over the entire voltage range. With Pb, the maximal current was generated with exactly the same depolarisation step (to −5 mV) as without Pb. All other metals shifted the maximum of the current voltage relation curve to more depolarized potentials. This shift depended upon the concentration of the metal used and was more pronounced at higher concentrations of the metals. The shift was most obvious with Al, smaller with Zn, Hg or MeHg [14,15].

3.2.5. Simultaneous application of different metals and internal application

When Pb, Zn or Al were applied simultaneously in the range of their IC_{50} values, additive actions on VACCCs were found, which were independent of the order of application [16]. With 2 cations in the external solution, VACCCs were reduced by 75% (± 9%), and were even further reduced when a third metal was added.

Al, applied extracellularly or intracellularly, on VACCCs reduced VACCCs independently [17]. When Al was applied simultaneously both inside and outside, the 2 effects were additive, suggesting that Al has both an external and an internal binding site.

Besides the direct effects on NACs and VACCs we found some other effects, which are not directly related to their actions on NACCs or VACCCs:

3.2.5.1. Changes of membrane currents. While Pb, Al and Zn did not change the membrane current when used in the above-mentioned concentration range (in which the VACCCs were reduced), the application of higher concentrations of Hg (≥2 μM) or MeHg (≥10 μM) resulted in an unidentified membrane current. Hg caused an inward current, while MeHg generated a biphasic current with a transient inward and a long-lasting outward component [9,15].

3.2.5.2. Effects on long-term potentiation. Pb and Al reduce the generation and maintenance of long-term potentiation (LTP) in vitro [18,19] and in vivo [19,20]. In a rat brain slice preparation, Pb and Al reduced LTP in a concentration-

dependent fashion. LTP was abolished with concentrations of 10 μM Pb or 100 μM Al.

4. Discussion

VACCCs and NACCs are sensitive to the metals tested. Both Pb and Al reduce NACCs through the receptor/channel complex; however, the concentrations of metal needed were relatively high and most of the effects were reversible. Preincubation with Pb resulted in a more pronounced reduction of the current and there was always a small, but relevant, portion of the current which was irreversibly blocked. This irreversible effect might be more relevant in regard to the neurotoxicity of Pb.

The metals Pb, Al, Hg, MeHg and Zn reduce the currents through VACCCs, but their actions differed with respect to time course, affected calcium channel subtypes, use dependence, effective concentration range, pH dependence, and screening effects at the surface of the membrane (seen in a shift of the current voltage relations).

How could this variety of actions on VACCCs be explained? Why do all metal cations not act in the same, or at least in a similar way? For the explanation the following possible mechanisms have to be considered (Fig. 1): (1) chemical peculiarities of the various metal species; (2) unspecific effects at the membrane (screening of surface charges); (3) specific effects: (a) at the membrane, (b) at the entrance to the channel, (c) within the channel; (4) intracellular changes (interactions with second messengers, etc.).

4.1. Chemical peculiarities of various metal species

The active forms of Pb and Zn in physiological solutions (pH = 7.2–7.3 and a chloride concentration of about 120 mM) are most likely Pb^{2+} and Zn^{2+}. More than or other metals the form of Al in aqueous solution depends on pH: the amount of Al^{3+} increased when the pH decreased (down to 6.7) and the concentration of $Al(OH)_4^-$ increased when the pH decreased.

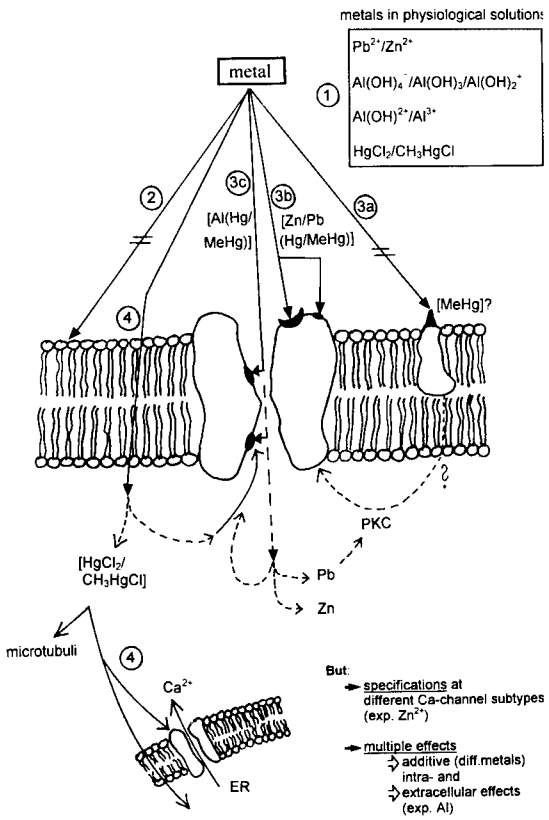


Fig. 1. Possible interactions of metals on voltage-activated calcium channel currents. For details see text.

Assuming, that Al^{3+} is the active form in reducing VACCCs, the pH dependence of the effect is easily explained. HG and MeHg probably exist in physiological solutions as uncharged complexes ($HgCl_2$ or CH_3HgCl), which might pass through the neuronal membrane [21]. But we do not know how these compounds react with the surface charge of the cell membrane. There they might very well exist as singly or doubly charged cations.

4.2. Unspecific effects at the cell membrane

Except for Pb, the metals tested shifted the maximum of the current-voltage relation for the

VACCCs concentration dependently to more depolarized voltages, the degree of shift depending on concentrations. Such a shift is typical of a charge screening effect. But since all concentrations used in our studies were in the micromolar range (even Al was never used in concentrations over 300 μM), a general screening of the surface charges at the cell membrane seems unlikely. Furthermore, a charge screening effect should change the currents through all channels in a similar fashion.

4.3. Specific effects

4.3.1. At the cell membrane

The shift of the current-voltage relation might be explained by specific binding sites at the surface of the membrane. But this is also improbable because this explanation suggests different specific binding sites for the different metals – otherwise the additive effects could not be explained – and the effect should be similar for all types and subtypes of voltage-activated channels (like a general charge screening effect), which is clearly not true.

4.3.2. At the entrance to the channel

Assuming that metal cations screen specific charges at the entrance of the channel, a specific metal binding site at this location could explain both the shift of the current voltage relation and the low concentrations needed. But such an explanation is only valid for such metal cations as Pb or Zn which do not need an open channel state for their action. The hypothesis that the binding site of these 2 cations might be at the entrance of the channel is underlined by another fact: compared to the other metals tested, the effects of these 2 cations were – at least partially – reversible.

4.3.3. Within the channel

For actions within the channel the metals must enter the channel. A binding site within the channel might be the main location of action of Al: Al needs an open channel to reduce VACCCs and its effects were not reversible. These facts indicate a strong binding site within the channel, which is not easily accessible. However, Al also produces a concentration-dependent shift of the current voltage-relation to more depolarized voltages, which indicates an additional screening of membrane charges due to binding at unspecific or specific sites. Binding of Hg and MeHg within the channel is also conceivable, because at least a part of the effects of these 2 compounds on VACCCs needed an open channel and the effects were not reversible.

4.4. Intracellular changes

Although we have no direct evidence for such mechanisms, we cannot exclude the possibility that some of the metals tested had internal actions which might have changed the currents through NACs or VACCs. Externally applied Pb reduces the rise of the internal calcium concentration without passing through the cell membrane [22]. In the case of Al we have a different reason for believing that it does not pass through the cell membrane; the additional reduction of the current when Al was applied intracellulary suggests not only a second effect, which is triggered intracellulary, but also demonstrates that there was probably no metal within the cell before it was added intracellularly.

Hg and MeHg are uncharged compounds in physiological solutions which are able to pass through the cell membrane and raise the internal calcium concentration [23], however, the time scale of such action is not known. Nevertheless, the first site of action of acutely applied Hg compounds is the surface of the cell membrane. We do not know to what extent the membrane currents we have seen at higher concentrations of Hg are related to intracellular effects.

The current through the NMDA receptor channel complex is reduced by Pb and Al. Pb, Hg, MeHg, Al and Zn reduce VACCCs. While the rise of intracellular calcium is most important for learning and memory, a change in the rise time and/or amount of calcium in the neuron might explain some of the long-lasting effects of these metals. For a specific metal ion we may be able to determine a mode of action which is more likely than other interactions, but we are not able to exclude most of the other possible external or internal mechanisms discussed above.

Overall, it is likely that the neurotoxicity of metal ions is mediated by a variety of different mechanisms. These different mechanisms and the simultaneous action of different metals at the same time are the reason for the complex neurotoxicity of metals on NACCs or VACCCs.

Acknowledgements

Due to restrictions of space not all important work is cited. This paper basically reviews some of our own data. Important contributions on the actions of metals on calcium channels have been made by other groups and I apologize to all my colleagues who are not mentioned. For critical reading I thank Dr. S. Cleveland and for technical assistance C. Wittrock, T. Kordela and P. Schwarz.

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Molecular mechanism of the lead-induced inhibition of rod cGMP phosphodiesterase

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Abstract

Retinal cGMP hydrolysis is inhibited following developmental and in vitro lead exposure. To determine whether Pb^{2+} directly inhibits the rod-specific cGMP phosphodiesterase (PDE) and to examine the kinetic mechanism of this inhibition, purified bovine rod cGMP PDE was assayed at varying [cGMP], [Mg^{2+}] and [Pb^{2+}]. Increasing [cGMP] or [Mg^{2+}] shifted the Pb^{2+} curves leftward or rightward revealing increased or decreased potency of Pb^{2+} (nM to pM range), respectively. A 1/velocity vs. 1/Mg^{2+} plot revealed that picomolar [Pb^{2+}] competitively inhibited PDE relative to millimolar [Mg^{2+}]. These novel findings reveal that Pb^{2+} binds at the Mg^{2+} site but with 4–6 log units higher affinity, thus preventing cGMP hydrolysis. These results may have implications for other enzymes using Mg^{2+} as a co-factor and suggest that Mg^{2+} may be useful for reversing the PDE inhibition by Pb^{2+}.

Keywords: Lead; Magnesium; Retina; Rod photoreceptor; Phototransduction; cGMP phosphodiesterase

1. Introduction

In vivo and in vitro lead exposure produce electrophysiological, biochemical and pathological alterations in the scotopic (rod-mediated), but not photopic (cone-mediated), visual system (see reviews [1,2]). For example, long-term rod-mediated visual deficits following low, moderate or high level developmental lead exposure have been reported in monkeys and hooded rats: peak blood lead values of 20, 30–60 and >60 μg/dl, respectively. Electroretinographic (ERG) studies conducted on these lead-exposed animals revealed long-term selective rod-mediated decreases in absolute and relative sensitivity, amplitude and temporal resolution and increases in latency and time to dark adaptation. Similar changes occur in isolated retinas following exposure to micromolar concentrations of lead chloride (see reviews: [1,2]).

Retinal biochemical studies have shown that developmental lead exposure produces long-term concentration-dependent decreases in retinal cGMP hydrolysis resulting in elevated levels of cGMP in both the dark-adapted and light-

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adapted states [3,4]. The inhibition of retinal cGMP hydrolysis was also produced by in vitro exposure to submicromolar concentrations of Pb^{2+} ([3], this paper). Presumably, these decreases in cGMP hydrolysis reflect a decrease in rod cGMP phosphodiesterase (PDE) activity.

This report will present: (1) biochemical data on the in vitro effects of Pb^{2+} on retinal cGMP hydrolysis; (2) biochemical data describing the molecular mechanism of inhibition of Pb^{2+} on rod cGMP PDE; and (3) a kinetic model for understanding the cGMP results. A detailed discussion of the relationship between the ERG results and alterations in cGMP metabolism was presented previously [5].

2. Materials and methods

2.1. Experimental animals and treatment

All experimental and animal care procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985). Animal care and maintenance and the lead exposure protocol for Long-Evans hooded rats were described previously [3,4]. Dark-adapted, frozen bovine retinas were from Hormel (Hormel, MN).

2.2. Materials

The chemicals and materials utilized, sources as well as their preparation have been described previously [3,6,7].

2.3. Cyclic nucleotide metabolism studies

Retinal [cGMP] and cGMP PDE specific activity were assayed in whole retinas obtained from control and lead-exposed rats or in control rat retinas following the addition of 10^{-9}–5×10^{-4} M free Pb^{2+} ([3], unpublished data). The specific activity of purified, trypsin-activated cGMP PDE, isolated from frozen dark-adapted bovine rod outer segments, was assayed following the addition of 5×10^{-11}–10^{-7} M free Pb^{2+} [6]. All assays were conducted in triplicate.

2.4. Cyclic GMP, Mg^{2+} and Pb^{2+} concentrations

PDE assays were conducted using measured total [cGMP] and computed and/or measured free [Mg^{2+}] and [Pb^{2+}] that encompassed the physiological and pathophysiological range to which rods might be exposed in vivo as discussed previously [7,8].

2.5. Statistical and graphical analysis

All group data were analysed using the appropriate analysis of variance (ANOVA) and post hoc multiple comparisons using Tukey's Honestly Significant Difference test according to the procedures provided by the SAS statistical package (SAS Institute Inc.; Cary, NC). For all data, the difference from controls was regarded as significant if $P < 0.05$. Graphical analysis utilizing double reciprocal plots were performed to determine various kinetic constants [9]. The half-maximal inhibitory concentrations (IC_{50}) of Pb^{2+} was determined using non-linear least squares analysis. Because double reciprocal plots deviate from linearity at extremes, only data lying between 10 and 90% maximum enzyme activity were used to determine apparent $K_{0.5}$ values.

3. Results

3.1. Whole retinal cyclic nucleotide metabolism studies

Prior exposure to lead during development resulted in significant dose-dependent increases in the retinal [cGMP] in both light- and dark-adapted rats [3,4]. In the 0.02% group, cGMP levels increased 12–19% whereas in the 0.2% group they increased 24–39%. The lead-induced increases in retinal [cGMP] resulted from an inhibition of cGMP hydrolysis (0.02%: −15%; 0.2%: −39%) since guanylate cyclase activity was unchanged.

In vitro studies examined whether the effects

of lead on cGMP PDE were direct ([3], this paper). cGMP hydrolysis was measured in homogenates of adult control retinas incubated with 10 μM cGMP, 1.5 mM Mg^{2+} and various [Pb^{2+}]. As illustrated in Fig. 1, the cGMP hydrolysis was inhibited significantly at [Pb^{2+}] $\geq$ 100 nM (−10% at 100 nM to −53% at 500 μM free Pb^{2+}). The IC_{50} value of Pb^{2+} was 1.1 ± 0.1 μM.

3.2. Interactions of cGMP, Mg^{2+} and Pb^{2+} with rod cGMP PDE

To determine if Pb^{2+} directly inhibited the rod cGMP PDE, initial studies were conducted using

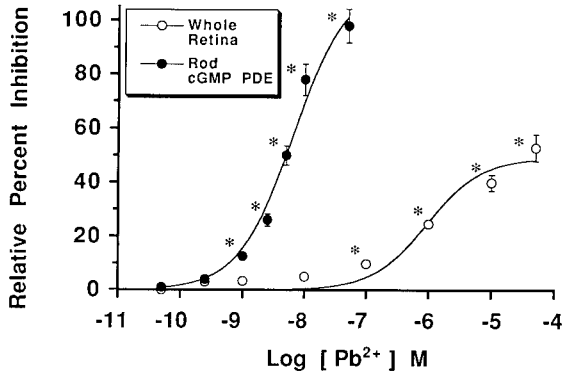


Fig. 1. In vitro exposure to picomolar to micromolar concentrations of free Pb^{2+} inhibits cGMP hydrolysis using adult control rat retinas and isolated, purified bovine rod cGMP PDE. All data have been normalized and are presented as percent inhibition relative to control (no Pb^{2+}). Each adult control whole rat retina was incubated with 10 μM cGMP, 1.5 mM Mg^{2+} and the indicated concentration of Pb^{2+} for 5 min as described in the Materials and methods section. Control activity was 11.0 ± 1.5 nmol cGMP hydrolyzed $(min)^{-1}$ $(mg\ protein)^{-1}$. Rod photoreceptor cGMP PDE (50 pM) was assayed using 5 μM cGMP, 500 μM Mg^{2+} and the indicated concentration of Pb^{2+} for 5 min as described in the Materials and methods section. Control activity was 21.09 μmol cGMP hydrolyzed $(min)^{-1}$ $(mg\ protein)^{-1}$. In either preparation, Pb^{2+} alone did not promote the hydrolysis of cGMP. Values represent the mean ± S.E.M. of 3–6 determinations for each data point. Curves were fit using non-linear least squares hyperbolas. The IC_{50} values of Pb^{2+} for the whole retinal and rod cGMP PDE were 1.1 ± 0.1 μM and 7.86 ± 1.09 nM, respectively. Mean values in the Pb^{2+} groups marked by an asterisk (*) were significantly different from controls at $P < 0.05$.

physiologically relevant concentrations of Mg^{2+} (500 μM) and cGMP (5 μM). Under this condition, picomolar to nanomolar [Pb^{2+}] significantly inhibited the isolated rod PDE with an IC_{50} value of 7.86 ± 1.09 nM (Fig. 1; Table 1).

To determine whether the Pb^{2+}-induced inhibition of PDE was affected by the [cGMP], assays were conducted using 1 μM to 1 mM cGMP, 10 μM to 10 mM Mg^{2+}, 50 pM to 100 nM Pb^{2+} and rod cGMP PDE. As cGMP increased from 1 μM to 1 mM, the Pb^{2+} concentration-response curves shifted to the left (Fig. 2A). Statistically significant inhibition of PDE activity was detected at lower [Pb^{2+}] (50 vs. 250 pM) when PDE was assayed in the presence of higher [cGMP] (1 mM vs. 1 or 5 μM). The IC_{50} values of Pb^{2+}, at each [Mg^{2+}], decreased (71–94%) as the [cGMP] was increased (Table 1). Moreover, for the same [Pb^{2+}] the extent of inhibition was significantly greater in the presence of higher [cGMP]. Even at the lowest [cGMP], PDE activity was inhibited ~100% by increasing the [Pb^{2+}]. Thus, and important from a pathophysiological standpoint, as the [Pb^{2+}] and [cGMP] increase in retinas of rats exposed to lead during development [3,4], the PDE inhibition would be expected to increase.

To determine whether the Pb^{2+}-induced inhibition of cGMP PDE was affected by the concentration of the co-factor Mg^{2+}, assays were conducted as a function of [Mg^{2+}]. The degree of the Pb^{2+}-induced inhibition of cGMP PDE was dependent on the [Mg^{2+}] (Fig. 2B). As the [Mg^{2+}] was increased, the concentration-response curves for Pb^{2+} shifted to the right. This is consistent with the significant increase in IC_{50} values of Pb^{2+} (9–55-fold) by Mg^{2+} at each [cGMP] (Table 1).

3.3. Kinetic analysis and mechanism of the Pb^{2+}-induced inhibition of rod cGMP PDE

Next, the nature of the Pb^{2+}-induced inhibition of rod cGMP PDE was determined. PDE was assayed using 5 μM cGMP, the indicated [Mg^{2+}] and either no Pb^{2+}, 750 pM Pb^{2+} or 1 nM Pb^{2+} (Fig. 3). A double reciprocal plot of the

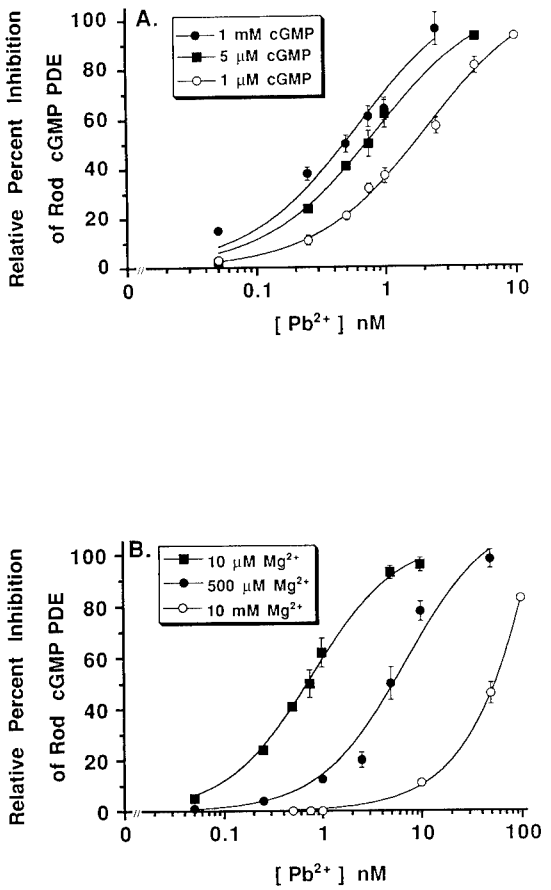


Fig. 2. cGMP increases and Mg^{2+} decreases the percent inhibition of the rod cGMP PDE by Pb^{2+}. (A) Rod photoreceptor cGMP PDE (50 pM) was assayed using 10 μM Mg^{2+} at the indicated concentrations of cGMP and free Pb^{2+}. All data have been normalized and are presented as percent inhibition relative to control (no Pb^{2+}). Control activity with: 1 mM cGMP was 26.69, 5 μM cGMP was 9.46, and 1 μM cGMP was 1.82 μmol cGMP hydrolyzed $(min)^{-1}$ (mg protein)$^{-1}$. Each data point represents the mean ± S.E.M. for 3–5 separate experiments. Curves were fit using non-linear least squares hyperbolas. The IC_{50} values of Pb^{2+} are presented in Table 1. (B) Rod photoreceptor cGMP PDE (50 pM) was assayed using 5 μM cGMP at the indicated concentrations of free Mg^{2+} and Pb^{2+}. All data have been normalized and are presented as percent inhibition relative to control (no Pb^{2+}). Control activity with: 10 mM Mg^{2+} was 23.66, 500 μM Mg^{2+} was 21.09, and 10 μM Mg^{2+} was 9.46 μmoles cGMP hydrolyzed $(min)^{-1}$ (mg protein)$^{-1}$. Lead alone did not promote the hydrolysis of cGMP. Each data point represents the mean ± SEM for 3–5 separate experiments. Curves were fit using non-linear least squares hyperbolas. The IC_{50} values of Pb^{2+} are presented in Table 1. Figs. 2A and B are reprinted by permission of the publisher from Srivastava et al. [7].

data revealed that the apparent $K_{0.5}$ of Mg^{2+} increased 2–3-fold in the presence of increasing [Pb^{2+}] (from 16 to 55 μM Mg^{2+}) while the apparent V_{max} was unchanged. This result demonstrated that Pb^{2+} is a competitive inhibitor of the rod cGMP PDE relative to Mg^{2+}.

Previously, the kinetics of cGMP hydrolysis by the rod cGMP PDE was analysed [6]. Briefly, cGMP and Mg^{2+} bind to the PDE in a rapid equilibrium random order reaction and ultimately form a ternary complex with the PDE. This ternary complex then produces the product, 5′GMP. Using this data and model, in conjunction with additional experiments performed with various concentrations of cGMP, Mg^{2+}, Pb^{2+} and IBMX (a competitive inhibitor of PDE relative to cGMP), a detailed kinetic analysis of the effects of Pb^{2+} was performed. These experiments allowed the generation of secondary and tertiary double reciprocal plots from which binding constants for each step of the reaction were derived [9]. These plots also resulted in the derivation of binding constants for the steps involved with the Pb^{2+}-induced inhibition of the rod PDE [6,7]. In Fig. 4, the proposed mechanism of the Pb^{2+}-induced inhibition of cGMP is presented. Lead directly inhibited the PDE by competing with Mg^{2+} for the metal site and this inhibition was dependent on the [cGMP]. Therefore, Pb^{2+} may substitute for Mg^{2+} and ultimately form a ternary complex with cGMP and the PDE. This ternary complex will not result in the formation of the product, 5′GMP, since Pb^{2+}, in the absence of Mg^{2+}, did not promote hydrolysis of cGMP. Consistent with the nature of competitive inhibitors, the addition of Mg^{2+} completely reversed the Pb^{2+}-induced inhibition of PDE as evidenced by the return of the rate of cGMP hydrolysis to the control rate (data not shown; see [7]).

4. Discussion

Retinal cGMP hydrolysis was inhibited in rats exposed to low to moderate levels of lead during development and in retinas acutely exposed to

Table 1
Half-maximal inhibitory concentrations (IC_{50}) of Pb^{2+} (nM) for the rod cGMP PDE[a]: effects of cGMP[b] and Mg^{2+}[c]

	10 μM Mg^{2+}	500 μM Mg^{2+}	10 mM Mg^{2+}
1 μM cGMP	2.13 ± 0.17	12.23 ± 2.55	67.0 ± 14.0
5 μM cGMP	0.80 ± 0.07	7.86 ± 1.09	44.0 ± 4.5
1 mM cGMP	0.45 ± 0.07	0.80 ± 0.05	3.86 ± 0.44

[a] The IC_{50} values of Pb^{2+} represent the mean ±S.E.M. of triplicate samples from 3–5 separate experiments. Values are reprinted by permission of the publisher from Srivastava et al. [7].
[b] At each concentration of cGMP, the mean IC_{50} values of Pb^{2+} at each concentration of Mg^{2+} were significantly different from each other at $P < 0.05$.
[c] At each concentration of Mg^{2+}, the mean IC_{50} values of Pb^{2+} at each concentration of cGMP were significantly different from each other at $P < 0.05$.

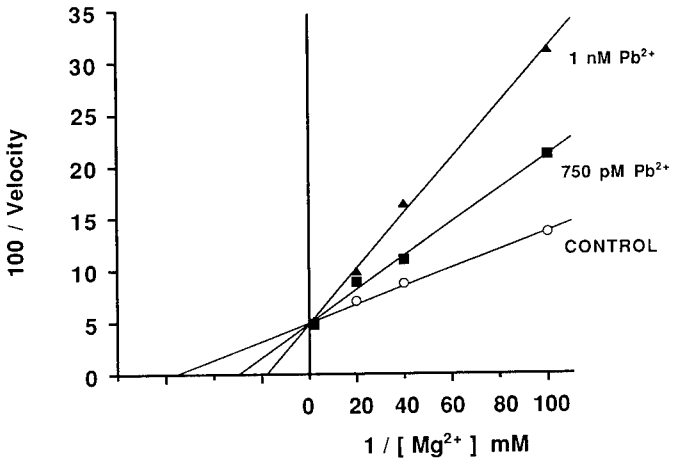


Fig. 3. Lead is a competitive inhibitor of the rod cGMP PDE relative to Mg^{2+}. The results from assays using 50 pM of rod photoreceptor cGMP PDE, 5 μM cGMP, and no Pb^{2+}, 750 pM or 1 nM Pb^{2+} have been re-plotted as 100/velocity vs. $1/Mg^{2+}$. Velocity is presented as μmol cGMP hydrolyzed $(min)^{-1}$ $(mg\ protein)^{-1}$. Under these conditions, the PDE in the absence of added Pb^{2+} had an apparent $K_{0.5}$ of 16 μM Mg^{2+} that increased to 33 and 55 μM Mg^{2+} with 750 pM and 1 nM Pb^{2+}, respectively.

submicromolar concentrations of Pb^{2+} in vitro ([3,4], this paper). In addition, the findings presented herein reveal that picomolar to nanomolar [Pb^{2+}] directly inhibit isolated, purified trypsin-activated rod cGMP PDE [7]. These results are consistent with earlier ERG and biochemical studies and extend these investigations by identifying a molecular site and mechanism of action of Pb^{2+}. Moreover, these latter results occurred at [Pb^{2+}] that are probably present in the rods of lead-exposed animals during developmental or following in vitro Pb^{2+} exposure.

The relative difference between the Pb^{2+}-induced inhibition observed in the whole retina compared to the isolated rod PDE preparation presented in Fig. 1 may reflect the decreased concentration of available Pb^{2+} due to significant non-specific binding of Pb^{2+} [10], the accessibility of sufficient amount of Pb^{2+} to the PDE and thus the free [Pb^{2+}] in the rod, and/or the [Mg^{2+}] (1.5 mM vs. 500 μM) used in the different experiments (retinal vs. isolated PDE). Interestingly, in a separate experiment, when the free [Mg^{2+}] was increased from 500 μM to 1.5 mM Mg^{2+} the PDE inhibition decreased from 98 to ~50% (data not shown; however, compare 500 μM and 10 mm Mg^{2+} data in Fig. 2B) resulting in the same degree of inhibition observed in the whole retinal experiment. Alternatively, the dif-

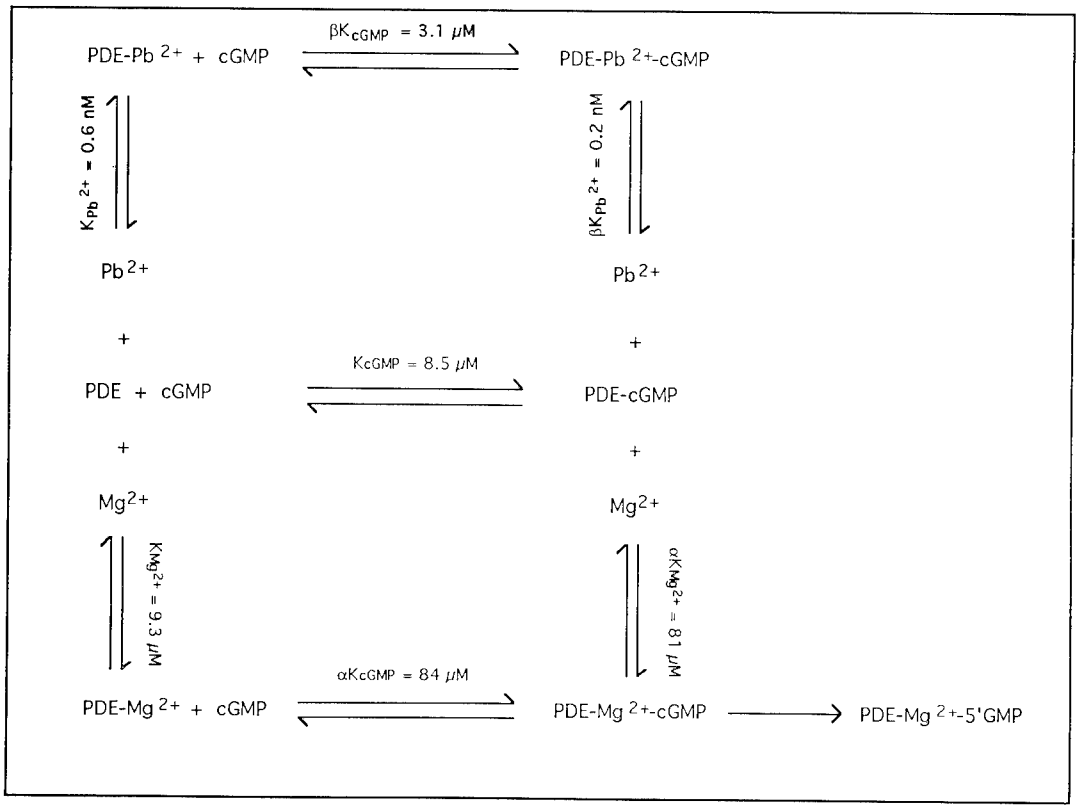


Fig. 4. Proposed mechanism of the Pb^{2+}-induced inhibition of cGMP hydrolysis. The hydrolysis of cGMP by the rod cGMP PDE may be modeled with a random binding order of Mg^{2+} and cGMP to the PDE [6]. Based on the results from Fig. 3, Pb^{2+} may substitute for Mg^{2+} and ultimately form a ternary complex with cGMP and the rod PDE. This ternary complex will not produce the product, 5'GMP. Fig. 4 is reprinted by permission of the publisher from Srivastava et al. [7].

ferences could have been due to the presence of the inhibitory PDE subunits in the whole retinal experiments [11] relative to the trypsin-activated PDE. This seems unlikely, however, since histone-activated PDE exhibited the same degree of Pb^{2+}-inhibition as trypsin-activated PDE (Srivastava, Hurwitz and Fox: unpublished data).

Since the potency of Pb^{2+} was affected by each co-reactant, Pb^{2+} must exert its effect within the catalytic pocket. The nature of the inhibition was confirmed by analysing the data with a double reciprocal plot which demonstrated that Pb^{2+} was in direct competition with Mg^{2+}-dependent activation of the PDE. This finding is intriguing since the hydrated ionic radius of Pb^{2+} is almost twice that of Mg^{2+} [12]. Although the exact molecular site of action responsible for the competitive inhibition is unknown, one of the following proposed mechanisms might explain this result. First, Pb^{2+} may bind at the same site as Mg^{2+}, but with higher affinity. Second, the binding site may be distinct from, yet sufficiently proximal to, the Mg^{2+} site to competitively inhibit Mg^{2+} from binding to the PDE [13]. Third, the binding of cGMP at the catalytic site may reduce the constraint surrounding the metal binding site on the PDE such that Pb^{2+}, but not Mg^{2+}, was better able to bind to the enzyme. This latter suggestion seems unlikely, however, since the binding order of divalent cations and cGMP to the PDE is random [6].

The inhibition of the PDE by Pb^{2+} was also sensitive to the [cGMP] since increasing concentrations of cGMP shifted the entire Pb^{2+} concentration-response curve to the left. That is, cGMP increased the potency of Pb^{2+}. This is

consistent with the higher affinity of Pb^{2+} for the PDE-cGMP complex when compared to the affinity of Pb^{2+} for the free enzyme (Fig. 4).

The IC_{50} values of Pb^{2+} ranged from 0.45 to 67 nM depending on the concentrations of Mg^{2+} and cGMP. Under physiologically relevant conditions, 1 nM Pb^{2+} significantly inhibited the rod PDE and the IC_{50} values of Pb^{2+} ranged from 7 to 12 nM. Under pathophysiological conditions, such as those encountered in lead-exposed rats with elevated levels of retinal cGMP [3,4] and possible lead-induced decrease in intracellular $[Mg^{2+}]$, as observed in other tissues [14,15], the IC_{50} may be as low as 800 pM Pb^{2+}. Thus, it is possible that a decrease in $[Mg^{2+}]$ and an increase in [cGMP] may occur simultaneously during in vivo lead exposure and thereby contribute to a significantly enhanced inhibition of PDE activity. These concentrations of free Pb^{2+} appear consistent with our animal model of low level lead exposure since $\geq$5 nM free Pb^{2+} would be found at a blood lead concentration of 10 μg/dl [16], which is considered toxic in children [17]. In our animal model, low level developmental lead exposure resulted in a peak blood lead of 19 μg/dl [4]. This suggests that an IC_{50} of 7–12 nM Pb^{2+} is consistent with the in vivo data.

The proposed reaction mechanism may apply to PDEs in general since the putative catalytic domain, where Pb^{2+} appears to exert its effect, is highly conserved among the entire family of PDEs [18]. One such PDE is the brain type IV cAMP PDE. The mutant 'dunce' gene in *Drosophila*, which codes for this brain cAMP PDE, is associated with defective memory and learning [19]. If this brain type IV cAMP PDE performs a similar function in vertebrates, then a direct inhibition of the brain type IV PDE by Pb^{2+} at the Mg^{2+} site may contribute to the long-term central nervous system deficits produced by lead exposure during neonatal development [20]. Thus, Mg^{2+}, which mediates the Pb^{2+}-induced inhibition of PDE, may be useful in reversing the inhibition produced by Pb^{2+}.

The findings of this study provide a novel mechanism for understanding the Pb^{2+}-induced inhibition of cGMP PDE. The results suggest that the direct inhibition of the rod PDE by Pb^{2+} may, in part, account for the observed electrophysiological alterations associated with in vitro and in vivo lead exposure (see reviews: [1,2]). Furthermore, the lead-induced inhibition of PDE and resultant elevation of cGMP may partially contribute to the apoptotic rod and bipolar cell death observed in neonatal rats during the lead exposure period [1,21,22].

Acknowledgements

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Influence of toxicants on neural cell adhesion molecule-mediated neuroplasticity in the developing and adult animal: persistent effects of chronic perinatal low-level lead exposure

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Abstract

The expression of neuroplastic neural cell adhesion molecule (NCAM) polysialylated neurons in the dentate of juvenile (postnatal day 40) and adult (postnatal day 80) rats exposed to low-level lead during the early postnatal period has been investigated. At both ages, the number of polysialylated neurons was decreased significantly in lead-exposed animals when expressed per unit area but not total dentate area. This could be attributed to an increase in the number and intercellular spacing of granule cells in the dentate of the lead-exposed animals. These effects are related to NCAM polysialylation dysfunction perturbing early hippocampal neurogenesis.

Keywords: Polysialylation; Learning; Dentate; Proliferation

1. Introduction

The mechanisms which regulate neural development are dependent, in part, on time- and tissue-modulations of cell-cell and cell-substrate recognition systems which operate by homo- or heterophilic interactions [1]. Based on structural and functional considerations, they may be grouped into 3 major families – the integrins, cadherins and the immunoglobulin (Ig) superfamily. The neural cell adhesion molecule (NCAM) is the best characterised member of the Ig superfamily. The expression of NCAM is regulated dramatically during development with respect to isoform expression and post-translational modification of the polypeptide core. The dominant neural NCAM forms are obtained by the alternate splicing of a single gene and comprise 3 polypeptides of 180, 140 and 120 kDa. NCAM glycosylation state is unique and involves post-translational additions of α2,8-linked polysialic acid (PSA) homopolymers which can exceed 55 sugar units in length. Recent evidence suggests that modulation of NCAM prevalence and polysialylation continues in defined brain regions in the adult which retain neuroplastic potential such as the hippocampus, hypothalamic-neurohypophysial axis and olfactory bulb [2].

The molecular mechanisms by which NCAM regulates neural structuring remain to be established. Antibody interventives studies have suggested NCAM prevalence and polysialylation to be critical in neuritogenesis, neurite pathfinding and, later, in memory formation [3–5]. Poly-

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sialylation may be the critical functional feature for NCAM-mediated neural plasticity. Failure to down-regulate NCAM polysialylation in the period of final postnatal synaptic elaboration results in gross structural deficits as is observed in the cerebellum of the staggerer (*sg/sg*) mouse mutant [6]. Furthermore, mice homozygous for disrupted NCAM gene function do not have profound deficits in their overall development but exhibit learning deficits which may arise from memory-associated polysialylation deficits [7]. With increasing age NCAM polysialylation state declines dramatically until virtually none is detectable in the aged-brain when neuroplasticity is attenuated significantly [8].

Animal studies have demonstrated lead-induced neurobehavioural deficits to arise from perinatal exposure to threshold levels of 20 μg/dl blood (for review see [9]). At these concentrations of blood lead no marked abnormality in the neural structuring of areas such as the cerebellum, which is formed entirely during the early postnatal period, have been observed [10]. However, adult animals exposed to low-level lead from time of conception or birth to postnatal day 30 exhibit pronounced recall deficits following passive avoidance training [11] suggesting that the persisting cognitive and behavioural deficits must arise from a subtle dysfunction in the production and/or selection of synapses which are expressed transiently during early neural development.

Previously, we have demonstrated chronic low-level lead exposure to impair the expected decrease in NCAM polysialylation state in the period when final synapse formation is completed, an effect consistent with persisting abnormalities in connectivity pattern [12]. Given NCAM polysialylation state persists in the adult hippocampus and is integral to the neuroplastic events which underlie memory formation [2,4] it provides a basis to explore subtle persisting effects of perinatal lead exposure. The polysialylated neuronal structures comprise a distinct population of cells which reside at the border of the granule cell layer and hilar region [8]. During periods of consolidation following passive avoidance training the number of polysialylated neurons increase in a task-specific manner. This learning-induced increase in NCAM polysialylation state is believed to be required for the memory consolidation process of synapse formation and change in connectivity pattern [13], widely believed to underlie the memory process [14]. Thus persisting deficits in this polysialylated neuronal population may account for the memory consolidation deficits noted in adult animals exposed to chronic low-level lead during their early perinatal period.

2. Materials and methods

2.1. Lead exposure protocol

To explore the relationship between early low-level lead exposure and attenuated adult neuroplastic potential, we exposed Wistar rat pups, culled to 8 at birth, to 400 mg $PbCl_2$/l via their dams' drinking water from conception until postnatal day 30. This exposure protocol results in pup blood lead levels which range from 45 μg/dl on postnatal day 4 to 15 μg/dl on postnatal day 12 and, thereafter, increase to 40 μg/dl on postnatal day 20 [10,12]. At postnatal day 30 the lead was removed from the drinking water and the animals were allowed attain adulthood at which time blood lead levels ranged from 2–4 μg/dl. Adult dietary intake and body weights were similar in both the control and lead-exposed groups and no differences in pup whole brain/body weight ratio were evident at time of sacrifice.

Dissected whole brains were coated immediately in an optimal cutting temperature compound (Gurr, UK), snap-frozen in liquid nitrogen-cooled *n*-hexane and stored at −80°C until required for further processing. Horizontal sections of 12 μm were cut from frozen tissue using a MICROM (Series 500) cryostat. Serial sections were obtained for analysis from a point −5.6 mm from Bregma [15] and thaw-mounted onto 0.1% (w/v) poly-l-lysine coated glass slides.

The sections were fixed in 70% (v/v) ethanol for 30 min, washed twice for 10 min in a washing buffer of 0.1 M phosphate-buffered 0.9% saline (PBS), pH7.4, and incubated overnight (20 h) in a humidified chamber at room temperature with

anti-PSA [16] diluted 1:500 in an incubation buffer composed of PBS containing 1% (w/v) bovine serum albumen (Sigma Chemical Co., UK) and 1% (v/v) normal goat serum (DAKO, Denmark) in order to eliminate non-specific staining. The sections were washed again and exposed for 3 h to fluorescein-conjugated goat anti-mouse IgM (Calbiochem, UK) diluted 1:100 with incubation buffer. The sections received a final wash before being mounted in Citifluor® (Agar, UK), a fluorescence-enhancing medium.

The staining pattern was observed with a Leitz DM RB fluorescence microscope using an exciting wavelength of 495 nm and an emitting wavelength of 525 nm. Immunofluorescence staining was specific as it was eliminated completely by omission of either the primary or secondary antibody and by pre-absorbing anti-PSA with colominic acid (1 mg/ml; Sigma Chemical Co., UK), which contains α2,8 homopolymers of sialic acid (data not shown). Where relevant, sections were counter-stained by a brief exposure (60 s) to propidium iodide (50 ng/ml PBS) which was detected using an excitation wavelength of 552 nm and an emission wavelength of 570 nm. The total number of PSA-immunoreactive neurons in the dentate granule cell layer and at the hilar border were counted in 10 alternate 12-μm sections commencing −5.6 mm from Bregma, to preclude double counting of the 5–10 μm perikarya. Cell counts were divided by the total area of the granule cell layer, which included all propidium iodide-labelled cells, and multiplied by the average granule cell layer area which was 0.15 ± 0.01 mm^2 at this level, and the mean ±S.E.M. calculated. These means were used to establish the mean ±S.E.M. for each animal group. Area measurements of propidium iodide-stained granule cell perikarya were performed using a Quantimet 500 Image Analysis System.

3. Results

Juvenile postnatal day 40 rats, exposed to chronic low-level lead from time of birth to postnatal day 30, exhibited a marked attenuation in the number of polysialylated neurons/unit dentate area in the hippocampal granule cell layer-hilar border region as compared to their control counterparts (Fig. 1A and C). However, area analysis of propidium iodide-stained hippocampal sections revealed the dentate to be much larger in the lead-exposed populations. When corrected for the increased dentate size no difference was apparent in the number of polysialylated neurons between control and lead-exposed animal groups. Similar results were obtained in the adult postnatal day 80 animal (Fig. 1B). The increased area of the dentate persisted and the overall reduction in polysialylated neurons is attributable to their age-dependent decline [8].

The enlargement of the dentate was attributable to a marked change in width due to an increase in the number of granule cell rows and intercellular area (Fig. 1C). At postnatal day 40 the number of cells spanning the granule cell layer was 10.37 ± 0.22 in the control group as compared to 12.62 ± 0.96 ($P < 0.05$; unpaired Student's t-test) in the lead-exposed animals. A similarly significant difference was noted in the postnatal day 80 animals. Direct measurement of the granule cell layer demonstrated an approximate 20% increase in width in the lead-exposed group but this was not observed to persist in the postnatal day 80 animals.

An apparent dysmorphogenesis was observed in the ventral blade in the dentate gyrus in some of the lead-exposed animals (Fig. 1C). However this was not a consistent finding as the distance from the apex of the dentate gyrus to the end of the ventral blade was not significantly different in the lead-exposed animals when compared to their control counterparts.

4. Discussion

The persisting structural deficits observed in the hippocampal formation may be expected to contribute to the neurobehavioural deficits associated with early postnatal lead exposure as this brain region is intimately associated with memory formation [17]. The overall lead-induced change in dentate morphology was unexpected and, to our knowledge, no similar observation

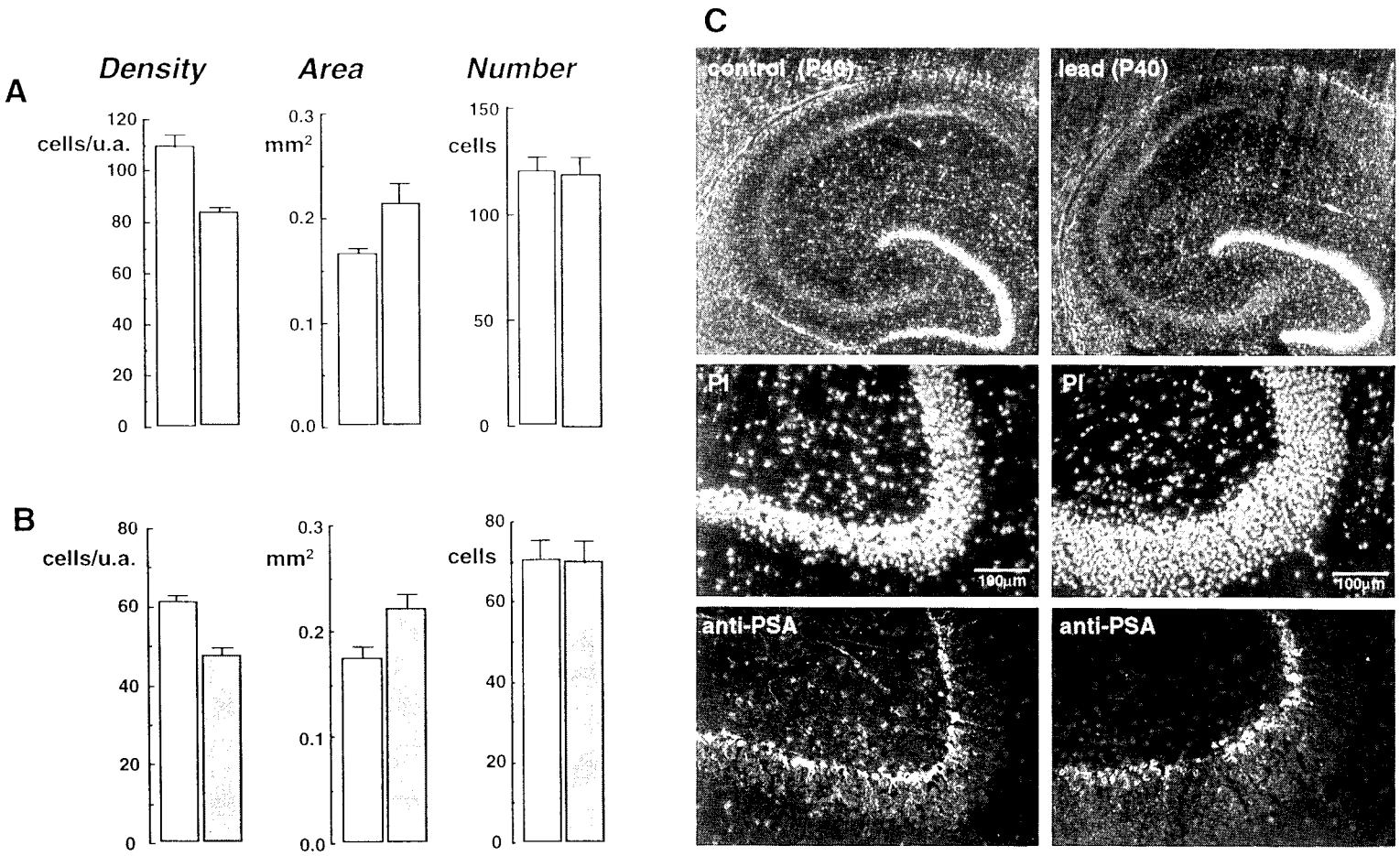


Fig. 1. Influence of postnatal lead-exposure on dentate polysialylated neurons and morphology in the juvenile and adult Wistar rat. Panels A and B illustrate the density of polysialylated neurons, dentate area and number of polysialylated neurons per section in control and lead-exposed animals at postnatal day 40 (A) and 80 (B), respectively. Values of the mean ±S.E.M. ($n = 7–8$). The propidium iodide (PI)-stained sections in panel C illustrate abnormal dentate morphology and increased granule cell layer width in the lead-exposed animals and the anti-PSA staining the position of the polysialylated neurons of the hilar/granule cell layer border.

has been reported. The profound increase in the number of neurons in the dentate is consistent with impaired proliferative control during early neural structuring as no increase in dentate area was observed in lead-exposed animals between postnatal day 40 and postnatal day 80.

Previous studies have demonstrated lead to lengthen the cell cycle during periods of cell acquisition in the developing cerebellum [10]. However, no dramatic change was noted in overall cell acquisition or in the migration of the granule cells from the external to internal cell layer. If the measured increase in DNA biosynthetic rate reflects a prolonged period of neuronal cell proliferation, then increased hippocampal dentate neurodevelopmental proliferation may be expected as lead accumulates preferentially into this brain region [18]. The increase in DNA biosynthetic rate persists until the period when NCAM polysialylation state becomes attenuated in the normal animal [10,12]. Given that NCAM can exert growth factor-independent inhibition of astrocyte proliferation [19], persisting neuronal and/or glial polysialylation may be expected to attenuate cell interaction and result in prolonged periods of neuronal and glial proliferation during development. Increased glial cell number and/or hypertrophy may account for the expanded areas of intercellular contact observed in the granular cell layer of lead-exposed animals [20,21].

Persistent NCAM polysialylation in the early perinatal period of lead-exposed animals does not appear to significantly influence the number of dentate polysialylated neurons in the juvenile and adult animal. Thus their full neuroplastic potential is retained but likely to be impaired significantly by the overall structural abnormalities observed in this brain region following perinatal low-level lead exposure. Although their position within the dentate remains unaltered the extent of dendritic arborisation required to extend to the molecular layer becomes significantly increased. As yet, it is unclear if these structural abnormalities will result in an attenuated activation of NCAM-mediated neuroplastic events during memory formation.

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Uptake and fate of ozone in the respiratory tract

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Abstract

Ozone (O_3) is a ubiquitous pollutant with an array of established effects following acute and chronic exposure. Absorption of O_3 occurs in all regions of the respiratory tract, but injury to the pulmonary region appears to be of greatest concern because of the susceptibility of this region to the development of chronic disease. Processes that affect the uptake and transport of O_3 and available dosimetry models are briefly reviewed prior to discussing recent experimental dosimetry data in laboratory animals and humans. Dosimetry model predictions are compared with experimental data, and an example is provided that illustrates the potential for such models to contribute to our understanding of toxicological results.

Keywords: Uptake; Ozone; Dosimetry models; Respiratory tract absorption; Gas transport

1. Introduction

Ozone (O_3) is a highly reactive, poorly water-soluble gas that is absorbed in all 3 major regions (extrathoracic, tracheobronchial, and pulmonary) of the respiratory tract of laboratory animals and humans. While this ubiquitous pollutant is formed photochemically in urban air from oxides of nitrogen and volatile organic compounds, transport long distances downwind from urban sources is commonplace [1]. In the United States, millions of people are exposed to levels of O_3 above the current National Ambient Air Quality Standard of 0.12 ppm. Although acute effects of O_3 have been documented in both laboratory animals and humans at or below the current standard, potential human health effects of O_3 following chronic, low-level exposure are of greater concern. This concern has arisen in part because of a recent study showing effects of O_3 in the centriacinar region of the lungs of rats exposed chronically to a near-ambient urban profile of O_3 [2] and from subchronic studies in nonhuman primates reporting effects in the comparable region of the lung from exposure to 0.15 ppm [3]. For a recent review of an array of acute and chronic effects of O_3 and their implications for standard setting, the reader is referred to Lippmann [4].

Understanding interspecies differences in the respiratory tract disposition of inhaled O_3 is critical in order to assess the implications of toxicological results from animal studies for human risk. Differences among species in O_3 uptake may or may not result in different O_3 doses delivered to target sites in the respiratory tract. Knowledge of dose enables exposure response data to be converted to dose-response relationships for the purpose of either intra- or interspecies extrapolation. Here, some of the major factors influencing the respiratory tract uptake of O_3 will be briefly discussed, and

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currently available experimental data on the uptake of O_3 in laboratory animals and humans will be reviewed. An example that illustrates the potential of O_3 dosimetry models to contribute to toxicological responses will also be discussed.

2. Factors affecting O_3 uptake

The uptake of O_3 in specific regions of the respiratory tract is determined by the complex interaction of a number of factors. Broadly defined, these factors relate to the structure of the given respiratory tract region, the nature of ventilation, and the mechanisms responsible for gas transport. Absorption is the term generally used when referring to the physical and chemical processes that result in removal of O_3 from the airstream. Here, uptake refers to the total mass of O_3 absorbed in a region compared to the mass entering that region and is usually expressed either as a percentage or as a fraction.

2.1. Respiratory tract structure

The extrathoracic (nose or mouth through the larynx), tracheobronchial (trachea through terminal bronchioles), and pulmonary (alveolar) regions make up the major subdivisions of the respiratory tract. The extrathoracic region is often termed the upper respiratory tract (URT), while the tracheobronchial and pulmonary regions combined are referred to as the lower respiratory tract (LRT). Although there are significant species differences in URT and LRT structure, these differences are much more pronounced for the URT. Gross anatomical structures comprising the URT do not scale according to body mass. The size and shape of the nose and the nasal turbinates are largely responsible for species differences in internal nasal anatomy [5]. These gross anatomical variations among rats, monkeys, and humans in the structure of the nose impart different major airflow streams in these species [6,7] and hence differences in local absorption of O_3 and potential for site-specific tissue damage. Since laboratory animals and humans have the same major epithelial cell types in the nose (squamous, transitional, respiratory, and olfactory), the net result of species differences in URT uptake of O_3 relates to the importance of establishing and understanding site-specific dose and response.

2.2. Ventilation

The route, depth, and rate of breathing influence the amount of O_3 inhaled. Results are available from various investigations [8–10] on the uptake of O_3 by the URT in human subjects breathing through the nose versus the mouth. Gerrity et al. [8] found a small decrease in O_3 uptake when breathing through the nose compared to the mouth that was statistically significant, but not likely biologically significant. Weister et al. [9] also concluded that in humans O_3 absorption is not different for the 2 routes of breathing. In experiments using a bolus inhalation system, however, Kabel et al. [10] found that only 50% of the inhaled O_3 was absorbed by the URT during oral breathing compared with 80% during nasal breathing. The possible influence of the nasal cannula and mouthpiece on their results was assessed by Kabel et al. [10]; they found no systematic changes that would weaken their findings.

The depth and rate of breathing determine minute ventilation that, in turn, is a function of overall metabolic rate and physical exertion levels. The lungs are designed to efficiently deliver oxygen to the gas-exchange region over a wide range of activity levels. Thus, a relatively insoluble gas like O_3 is carried deep into the lung even at normal levels of respiration. With heavy exercise, there is predicted to be a 10-fold increase in total mass uptake of O_3 in the human lung [11] with a disproportionate increase in pulmonary region uptake. A consistent finding in human clinical studies is that exposures at rest to O_3 concentrations greater than about 0.5 ppm or at exercise to levels below 0.2 ppm result in an increase in breathing frequency accompanied by a decrease in tidal volume such that minute ventilation remains relatively unchanged [12].

2.3. Gas transport mechanisms

Gas transport occurs in the airway lumen, liquid lining layers of the respiratory tract, tissue, and blood. Various mechanisms are operable to

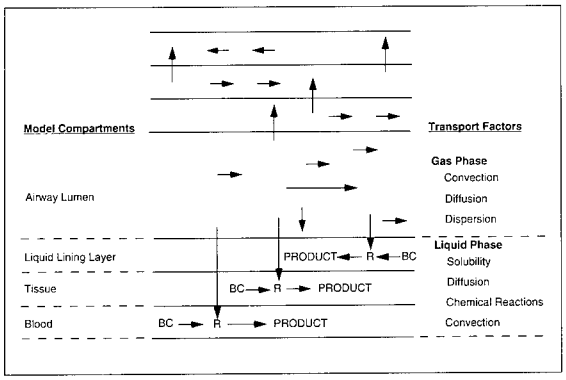


Fig. 1. Diagram illustrating potential compartments and transport factors that may need to be included in the formulation of an O_3 dosimetry model. The relative arrangement of the airway lumen, liquid lining layer, tissue, and blood compartments of the respiratory tract is shown. Since model compartments are symmetric about the airway lumen, only the lower half of the figure is fully labeled. R, chemical reactions between O_3 and biochemical constituents (BC); PRODUCT, the resulting products. Transport factors important in the various model compartments are noted. Modified from Miller and Kimbell [15].

differing degrees in the gas phase (airstream) compared with the liquid phase, as shown schematically in Fig. 1. Transport of O_3 in the gas phase is governed by convection, effective axial dispersion, and loss to airway walls. Convection refers to the bulk movement of material. Bulk airflow is the predominant mechanism for gas transport in the URT and major conducting airways of the LRT, while diffusion is the major transport mechanism in the pulmonary region of the lung. The joint effects of turbulence, secondary airflow patterns, and complex airway geometry can be assessed in the tracheobronchial region through the use of an effective axial dispersion coefficient [13]. The relative importance of these gas phase transport mechanisms varies among respiratory tract regions for a given level of ventilation in any species. For example, airflow is nonturbulent in the rat nose at any physiologic flow rate but may be highly turbulent in the human nose during exercise. Convection can contribute to gas transport in the pulmonary region during heavy exercise. For additional discussion of gas phase transport mechanisms and their importance for modeling the absorption of reactive gases such as O_3, the reader is referred elsewhere [14,15].

Liquid phase absorption of O_3 in a given region of the respiratory tract is influenced by the biochemical composition of the fluids and tissues comprising the region and by chemical reactions of O_3 with specific constituents of the fluids and tissues. Convection, diffusion, and the solubility of O_3 are also important factors in liquid phase absorption. Before penetrating to underlying tissue, O_3 may be depleted in the liquid layers lining the respiratory tract. A mucociliary layer protects the tissue from direct exposure in the URT and tracheobronchial regions, while a thin layer of surfactant lines the gas-exchange region. The surfactant layer does not contain many constituents that react with O_3 so that surfactants act primarily as a diffusional resistance layer relative to O_3 absorption. The mucous layer, however, contains amino acids and unsaturated fatty acids as well as various other moieties [16] that can react with O_3, thereby leading to a distinction between net and tissue O_3 dose for the URT and tracheobronchial regions. Further discussion of the factors and processes affecting liquid phase absorption of O_3 may be found in Miller et al. [17].

3. O_3 dosimetry models

As noted earlier, knowledge of local dose provides the critical link between exposure and response. For human risk assessment purposes, mathematical dosimetry models can be used to estimate human exposure scenarios needed to produce the same dose of O_3 at specific sites associated with responses in animal toxicological studies. Inherent in this approach is the need to develop dosimetry models that can take into account species differences in respiratory tract anatomy, ventilatory parameters, and the physicochemical properties of O_3. For many gases, the state of knowledge of toxicological effects is such that dose on a regional (macro) scale is sufficient. For O_3, however, the toxicological database can be considered to be available on the cellular (micro) scale.

Fortunately, the development of O_3 dosimetry

models for laboratory animals and humans [11,18–23] has kept pace or been ahead of the quantitation of effects of O_3 on the LRT. To date, only the model of Hanna et al. [21] has addressed URT dosimetry in humans in more than a cursory manner. No detailed treatment of site-specific dosimetry of O_3 in the URT of laboratory animals is currently available. Thus far, URT absorption of O_3 in animals has only been incorporated indirectly, either by adjusting the tracheal input concentration to reflect URT scrubbing efficiency (fractional removal) or by including the URT volume in the model structure but ignoring URT absorption. However, computational fluid dynamic modeling methods such as those of Kimbell et al. [24] will allow future O_3 dosimetry models to examine site-specific dose and response relationships in the URT of various species.

An important aspect of respiratory tract structure currently not captured in any dosimetry model for O_3 concerns the representation of the mucociliary layer in the URT and tracheobronchial regions. In these regions, this layer is comprised of an epiphase and an underlying hypophase. The cilia of ciliated cells are bathed in the watery fluid of the hypophase. Sufficient quantitative data have not been available to allow modelers to distinguish the epiphase from the hypophase. Effects on ciliated cells of monkeys have been reported for relatively low levels of exposure to O_3 [25], leading to speculation that O_3 dosimetry models underestimate the tissue dose of O_3 in these regions. One, however, cannot rule out the proposal that effects on ciliated cells are mediated through O_3 effects on cilia in the hypophase, and, if so, model predictions of tissue dose may indeed be correct.

An area that affects modeling uptake and transport of O_3 in the respiratory tract concerns identifying the most appropriate dose metrics for the toxicological effects of this pollutant in the extrathoracic, tracheobronchial, and pulmonary regions. Recent ozonalysis studies [26], particularly with unsaturated fatty acids, indicate that reaction of O_3 with these olefins produces hydrogen peroxide and aldehydes that may be reactive intermediates associated with or responsible for the ultimate toxicity of O_3. In addition, Pryor [27] used these data, other biological information, and O_3 physicochemical data to predict that O_3 per se cannot penetrate any liquid lining layer greater than about 0.1 μm thick. He cites 0.1 μm as the thickness of the lung-lining fluid barrier (i.e., the surfactant layer) in the lower airways and concludes "... that little or none of the O_3 can penetrate this layer to attack the cells below it, except where it is at its thinnest or is entirely denuded." The distribution of the surfactant layer, however, is very heterogeneous since the layer conforms to minor perturbations of the surface of Type 1 cells and only pools in crevices and over Type 2 cells (Dr. Robert Mercer, Duke University Medical Center, Durham, NC, pers. commun.). The surface area of Type 2 cells makes up only about 2% of the alveolar surface area [28]. Thus, the surfactant layer is on average about 0.02 μm thick over about 98% of the alveolar surface of the lung and may approach thicknesses between 0.1 and 0.2 μm over about 2% of the lung (Dr. Robert Mercer, pers. commun.).

Nevertheless, Pryor's [27] analysis and postulation that a cascade of reaction products are involved in O_3 toxicity raise the question as to what is the relevant dose metric for toxicity in the extrathoracic, tracheobronchial, and pulmonary regions. Potential O_3 dose candidates other than tissue dose would be total dose, liquid lining layer dose, and dose of toxic reactive intermediaries. In judging the implications and appropriateness of various dose metrics, one needs to keep in mind the following points: (1) the thickness of the liquid lining layer is highly variable throughout the respiratory tract; (2) the lining layer may be patchy in distal conducting airways; and (3) surfactant pools within individual alveoli during the breathing cycle so that a time- and location-dependent thickness may need to be used in calculating O_3 mass transfer rates in the gas-exchange region.

4. Experimental dosimetry data and model predictions

Given the extremely high reactivity of O_3, direct measurement of O_3 tissue dose was not possible until the development of experimental

approaches involving ^{18}O, a stable isotope of oxygen, and isotope ratio mass spectroscopy in the latter 1980s [29,30]. Tissue absorption, however, was assessed indirectly as early as 1972 in animal studies where O_3 was drawn unidirectionally through URT airways that had been surgically isolated [31]. In recent years, a number of in vivo dosimetry studies have been conducted in laboratory animals and in human subjects. Some of the major findings of these studies will be briefly discussed.

Total respiratory tract uptake of O_3 was examined in 3 strains of rats and in guinea pigs by Weister et al. [32]. These investigators found no dependence on strain of rat nor any difference between rats and guinea pigs, with uptake averaging 47%. Studies of total respiratory tract uptake in human subjects have used tidal volumes ranging from 500 to 1650 ml and breathing frequencies ranging from 7.5 to 35 bpm [8,23,33]. Despite this wide range of ventilatory parameters, total uptake ranged from 78 to 97%. Thus the respiratory tract of the rat is less efficient in removing O_3 from the airstream compared with humans. This further highlights the need to understand differences between these species in regional O_3 removal to make appropriate dosimetric adjustments before comparing potential similarities in toxicological effects.

To date, the only human studies in which URT and LRT uptake have been determined in the same individuals have been conducted by Gerrity and coworkers [8,33]. Table 1 summarizes their results for uptake during cyclical breathing or steady unidirectional flow. Previously, Miller et al. [34] showed their model of LRT O_3 absorption predicted net doses that fit the experimental data well for the steady unidirectional flow experiments of Gerrity et al. [8]. As noted by Gerrity et al. [33], however, the dosimetry model of Miller et al. [11] overpredicts uptake of O_3 in the lung for the type of cyclical breathing regimen they used.

As shown in Table 1, URT uptake is about 40% for either type of airflow. Uptake efficiency of the LRT is considerably higher for steady unidirectional compared with cyclical flow (<70% versus about 90%) and consequently so is total uptake. A major part of this apparent difference is due to the following. Gerrity et al. [8] used peak plateau concentrations to compute uptake in their unidirectional steady flow experiments. In their cyclical breathing experiments [33], however, uptake was computed by integrating concentration and flow during the breathing cycle. To better compare the experimental data from the 2 studies, Gerrity et al. [33] computed peak O_3 uptake efficiencies using the extrema of the analyzer output signal. This procedure yielded LRT uptake efficiencies that were quite comparable to those obtained in their 1988 study but URT uptakes were still different (19 vs. 40%). Gerrity et al. [33] interpreted the differences in URT uptake to inspiratory flows that were 2–4 times greater than in their earlier study, whereas they felt that the similarities in LRT uptake might have resulted from offsetting effects of higher flows and greater tidal volumes in their cyclic breathing experiments.

Since normal respiration is cyclical in nature,

Table 1
Uptake efficiencies in human subjects during steady unidirectional or cyclical flow

Flow	Tidal volume (ml/s)	Breathing frequency (bpm)	Uptake efficiency		
			URT	LRT	Total[c]
Cyclic[a]	1650	25	0.37	0.68	0.88
	1239	35	0.41	0.62	0.87
Steady[b]	832	12	0.41	0.93	0.97
	778	24	0.38	0.89	0.96

[a] Data from Gerrity et al. [33] with uptake computed by integration during cyclic breathing.
[b] Data from Gerrity et al. [8] with uptake computed during constant unidirectional flow. Mouth and nasal exposure data were pooled.
[c] Data include an URT contribution for exhalation equal to that of inspiration since the original publications did not include the contribution from URT uptake during expiration.

uptake efficiencies determined using this exposure mode should probably carry greater weight when comparing dosimetry model predictions with experimental data. One should also bear in mind the fact that complex dosimetry model formulations for O_3 absorption have required various assumptions (e.g., model parameter values, reaction kinetics), estimations (e.g., mass transfer values, scaling of airway dimensions), and extrapolations (e.g., biochemical composition data from one species used to represent that for another). These limitations actually are one of the strengths of dosimetry modeling because the models can easily be modified to incorporate new knowledge or data. For example, the bolus exposure studies of Hu et al. [23] enabled experimental derivation of mass transfer coefficients for localized absorption of O_3 in tracheobronchial airways. After incorporating these mass transfer coefficient data and making other improvements (e.g., adding oral and pharyngeal compartments) to the O_3 dosimetry model of Miller et al. [11], model predictions of total respiratory tract uptake were found to be in better agreement with the experimental data (Dr. John Overton, U.S. Environmental Protection Agency, Research Triangle Park, NC, pers. commun.).

Regional respiratory tract absorption of O_3 in rats was examined by Hatch et al. [35] using ^{18}O analysis methods. While the major focus was to determine uptake by the URT, the authors had to use a compartmental model to derive an estimate of uptake. From measurements of ^{18}O enrichment, they found that the total respiratory tract enrichment was distributed among the head, trachea, and lungs according to percentages of 49.3, 6.5, and 44, respectively. Then, using a series of equations relating percentages removed by each compartment during inhalation and exhalation, they found URT uptake to be 17.4% on inhalation with a standard error of 1.5%. Thus, uptake efficiency in the head of the rat is considerably lower than that in humans. LRT uptake cannot be derived from the data reported by Hatch et al. [35]. However, if tidal volume and breathing frequency data from Weister et al. [32] are used as inputs into the equation for LRT dose that Overton et al. [36] developed using these parameters, LRT uptake alone would exceed the value of 47% of Weister et al. [32] for respiratory tract uptake (Dr. John Overton, pers. commun.). Thus far, dosimetry models for the rat have based mass transfer coefficients on biochemical composition and chemical reaction kinetic data from humans. A recent synthesis [37] of published data on phospholipid composition, biosynthesis, and secretion in various species contains quantitative data for the rat that should eliminate the need for the species-equivalent biochemical composition assumption in future O_3 dosimetry modeling efforts.

5. Response data and dosimetry model predictions

Once dosimetry models are developed, they can be used to examine a wide variety of toxicological issues. Computer simulation models can predict dose for a range of exposure scenarios to assist in synthesizing results from a single experiment or from multiple studies [34]. In recent years, 3-dimensional reconstruction techniques have been used to greatly expand our knowledge of the structure of the pulmonary acinus in laboratory animals and humans [38]. The implications of this information for use in dosimetry models have been reviewed by Miller et al. [17]. An example of how dosimetry models can contribute to our understanding of toxicological results is discussed briefly below to illustrate where modeling and toxicology are likely headed in the future.

For some time, pathologists and toxicologists have noted that O_3 exposure results in differing degrees of injury in different centriacini of the same rat [39,40]. This patchy lesion effect is a hallmark of O_3 toxicity. Collectively, a series of studies [22,36,41] demonstrate that differences in tracheobronchial path length and acinar volume combine to impart significant variability in O_3 dose to ventilatory units in the rat lung. (A ventilatory unit is defined to be the collection of all alveoli arising from a single terminal bronchiole, which is about 1500 alveoli for the rat.) Mercer et al. [22] hypothesized that large ven-

tilatory units at the end of short tracheobronchial paths may be disproportionately affected at low O_3 exposure levels.

Initial attempts to address this issue were made by Pinkerton et al. [42] in a study in which rats were exposed to 0.98 ppm O_3 nocturnally for 3 months. Following exposure, bronchiolar-alveolar duct junctions (BADJ) were isolated, concentric arcs were formed at 100-μm intervals distally through tissue sections, and the change in alveolar wall and septal tip thickness was determined. Fig. 2 shows changes in these end points for O_3-exposed animals relative to controls as a function of distance from the start of the BADJ. Also shown in Fig. 2 is the ventilatory unit dose of O_3 predicted by Mercer et al. [22]. Similar curvilinear relationships can be seen for both changes in alveolar wall thickness and predicted dose proceeding distally into the ventilatory unit. Although changes in septal tip thickness do not mimic the fall in predicted dose as well as do changes in alveolar wall thickness, Miller and Kimbell [15] attribute this to the fact that the conical model segments used by Mercer et al. [22] do not reflect the degree of microanatomy represented by septal tips. Nonetheless, one can infer from Fig. 2 that the usefulness of such dosimetry models in extrapolating toxicological results is clearly established. Indeed, Pinkerton et al. [43] have taken another major step along these lines in a study of rats chronically exposed to 0, 0.12, 0.5, or 1 ppm O_3. These authors studied selected airway generations and pulmonary acini arising from short and long tracheobronchial paths and analyzed the degree to which bronchiolar epithelium extended beyond the BADJ into alveoli. Pinkerton et al. [43] found this effect to be concentration-dependent and site-specific, with ventilatory units arising from a short tracheobronchial path of the left lung in male rats being the most affected by O_3 exposure.

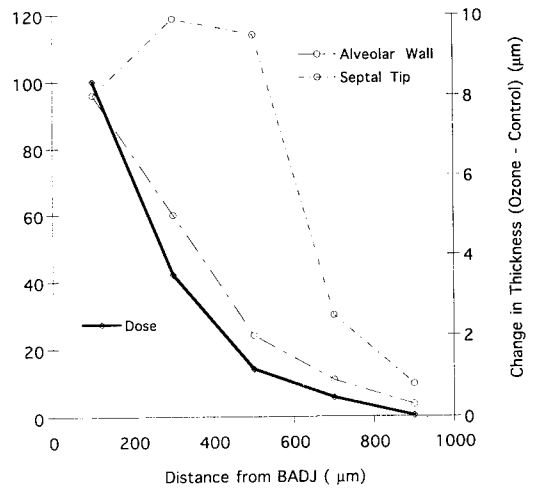


Fig. 2. The predicted dose of O_3 relative to distance from the BADJ and corresponding changes from control in alveolar wall and septal tip thickness. Rats were exposed nocturnally (8 h per night) for 90 days to 0.98 ppm O_3. Predicted dose changes are related to the left-hand y-axis; changes in alveolar wall and septal tip thickness are related to the right-hand y-axis. Reprinted with permission from Miller and Kimbell [15].

6. Conclusion

A wide array of responses have been shown in all major regions of the respiratory tract following acute and chronic exposure of laboratory animals to O_3. Pulmonary region effects appear to be of greatest concern, however, because of the susceptibility of this region to the development of chronic disease. An understanding of dose is critically important for linking exposure with response prior to using chronic toxicity data to assess potential human health risks from exposure to O_3. Major factors affecting O_3 uptake include the structure of the respiratory tract, the nature of ventilation, and the mechanisms of gas transport. Information on these factors was briefly reviewed as well as the general nature of O_3 dosimetry models that incorporate this information. Results from various experimental dosimetry studies on O_3 uptake in the respiratory tract were discussed, and predictions from O_3 dosimetry models were compared with some of the experimental findings. Lastly, an example was discussed that illustrates the potential for dosimetry models to contribute to our understanding of the toxicological responses associated with exposure to this ubiquitous air pollutant.

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A new mechanism for the toxicity of ozone

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Abstract

Ozone, with its high reactivity, is entirely consumed as it passes through the first layer of tissue it contacts at the lung/air interface. This layer includes the epithelial cell lining fluid (ELF) and, where the ELF is thin or absent, the membranes of the epithelial cells that line the airways. Thus the biochemical changes that follow the inhalation of ozone must be relayed into deeper tissue strata by a cascade of ozonation products. Lipid ozonation products (LOP) are suggested to be the most likely relay molecules of ozone's signal. This is because unsaturated fatty acids are present in relatively high concentrations in both the ELF and in pulmonary cell bilayers, and ozone reacts with unsaturated fatty acids to produce ozone-specific products. Further, LOP are finite in number, have structures that are predictable from the Criegee ozonation mechanism, and are small, diffusible, stable (or meta-stable) molecules, similar to other lipid-derived signal transduction species. Preliminary data show that individual LOP cause the activation of specific lipases, which trigger the release of endogenous mediators of inflammation.

Keywords: Ozone; Lipid ozonation product; Inflammation; Epithelial cell lining fluid; Lipase; Phospholipase

1. Introduction

The inflammatory effects of ozone have been demonstrated in both animal and human studies [1]; effects include airway hyperactivity, increased epithelial macromolecular permeability, neutrophil infiltration, and airway mucus hypersecretion [1–6]. There is strong evidence for the presence of lung inflammation several hours after exposure [1]. Despite the long history of the study of ozone, the molecular steps that cause this pathology are not known.

We here review the suggestion that the toxic effects of ozone are due, at least in part, to a cascade of lipid ozonation products (LOP) rather than to ozone itself [7,8]. Fig. 1 is a cartoon showing the proposed cascade mechanism.

The cascade mechanism originated in calculations that suggest that ozone is too reactive to penetrate far into tissue; only a very small fraction of the total dose of ozone can pass unreacted through a bilayer membrane, and none can pass through a cell [9]. Yet we know there are both pulmonary and extra-pulmonary effects of ozone, and so we must ask: how do these effects arise?

It is logical to propose that a cascade of ozonation products is responsible for the damage that occurs when ozone is inhaled. Ozone must react in the first layer of tissue it contacts at the lung/air interface. This layer includes the epi-

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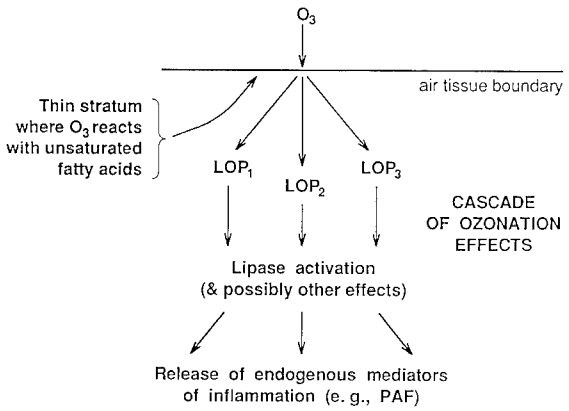


Fig. 1. A cartoon showing the cascade mechanism for ozone toxicity. Ozone reacts with lipids to form LOP; these LOP then activate lipases, and perhaps have other biological properties. The activated lipases result in the production and release of endogenous cellular signal transduction molecules such as eicosanoids, PAF, and others.

thelial cell lining fluid (ELF) and, where the ELF is thin or absent, the membranes of the epithelial cells themselves that line the airways. But, it must be recognized that the cascade mechanism is a 'Tinker-to-Evers-to-Chance' relay of a signal, and, as in baseball, the nature of the shortstop and second baseman is critical in determining the speed of the relay and the damage done. And so we must ask: what is the nature of the products that relay the effects of ozone?

For several reasons, we believe that LOP are the most likely transmitters of ozone's message. Firstly, the ELF is about 90% lipid and 10% protein, and the concentration of unsaturated fatty acids, and particularly mono-unsaturated fatty acids, in the lipids is appreciable. Table 1 lists the types of lipid that occur in human ELF; as can be seen, phosphatidylcholine is the principal lipid. Table 2 gives the percent of unsaturated fatty acids present in human ELF; phosphatidylcholine contains about 7% palmitoleic and about 15% oleic, plus small amounts of more highly unsaturated fatty acids.

Secondly, some ozone is destroyed by reaction with antioxidants such as vitamin E, ascorbate,

Table 1
Major phospholipids of human ELF and lung tissue

Phospholipid[a]	ELF[b] (%)	ELF[c] (%)	ELF[d] (%)	Lung tissue[e] (%)
Phosphatidylcholine	73	69	68	81
Phosphatidylglycerol	12	11	10	9
Phosphatidylethanolamine	3	8	5	2

The percentage composition refers to the surfactant isolated from minced lung tissue by repetitive centrifugation.
[a] The phospholipid fraction typically accounts for 70–90% of the total lipid [34].
[b] Ref. [35].
[c] Ref. [36].
[d] Ref. [37].
[e] Ref. [38].

Table 2
Percentage unsaturated fatty acids present in human ELF major phospholipids

Fatty acid	PC[a]	PE	PG
$CH_3(CH_2)_5CH=CH(CH_2)_7CO_2H$			
Palmitoleic acid	8, 5	4	5
$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$			
Oleic acid	17, 11	47	34
$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7CO_2H$			
Linoleic acid	1, 4	8	3
$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH-CH_2CH=CH(CH_2)_3CO_2H$			
Arachidonic acid	0, 2	5	1

[a] The first entry in this column is calculated from Ref. [36] and the second is from Ref. [35]. All other values are from Ref. [36].

and glutathione, but these reactions probably are part of a sacrificial protection system that leads to few toxic products. (They may, however, lead to signals of generalized oxidative stress such as depleted glutathione [10].) Furthermore, despite this protective screen of antioxidants, some ozone does react with lipids in the lung [8,11–16].

Thirdly, lipids give small, diffusible products upon ozonation, rather than the less defined products formed from proteins [17]. LOP are stable (or meta-stable) molecules with structures similar to known lipid-derived signal transduction species.

The ozonation of lipids gives products with structures that are predictable from the Criegee mechanism of ozonation; this mechanism is shown in Fig. 2. If ozonation occurs in a partly aqueous area, such as the ELF, then the ozonation process produces aldehydes, hydroxyhydroperoxides and small amounts of the Criegee ozonide.

The relative amounts of the products that are formed from the ozonation of lipids can be predicted from the known rate constants with which the double bond in the lipid reacts with ozone [18–21] and the relative amounts of the unsaturated fatty acids in the ELF and pulmonary membranes. Since, as we have seen, only a limited number of mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) occur in lung tissue, only a limited number of LOP will be formed.

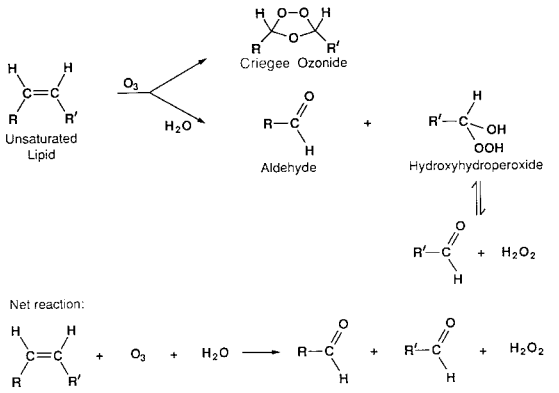


Fig. 2. The top portion of this figure shows the mechanism of ozonation of an olefin (such as an unsaturated fatty acid) that contains a *cis* double bond. Ozonation can produce either the Criegee ozonide or, if water is present, a molecule of aldehyde and one of a HHP. The HHP is in equilibrium with another molecule of aldehyde and hydrogen peroxide. The net reaction, therefore, gives 2 moles of aldehyde and 1 mole of hydrogen peroxide per mole of ozone and olefin used [39,40].

For example, the most prevalent MUFA in the ELF are palmitoleic (16:1($n-7$)) and oleic acids (18:1($n-9$)), which give heptanal and nonanal as their aldehydic products upon ozonation. Furthermore, these aldehydic products are relatively specific to ozonation, since MUFA do not undergo autoxidation (a process that also can produce aldehydes). Fig. 3 outlines possible LOP from various types of pulmonary lipid.

2. Structures of the LOP

Note that ozonation of an olefin in the presence of water can give rise to either the Criegee ozonide or to the fragmentation of the substrate into 2 different pairs of aldehydes and hydroxyhydroperoxides [22]. For example, if the olefin is a lipid such as 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylcholine (POPC), then the LOP shown in Fig. 4 are formed. These LOP include the POPC Criegee ozonide (POPC-Oz) and 2 aldehyde-hydroxyhydroperoxide pairs, one in which the aldehyde is attached to the glycerol backbone (PC-Ald) paired with a 9-carbon hydroxyhydroperoxide (HHP-C9), and where the hydroxyhydroperoxide is attached to the glycerol backbone (PC-HHP) and a 9-carbon aldehyde (Ald-C9; nonanal) is released. Thus, 5 principal LOP are formed from POPC (if stereoiosomers are ignored). As can be seen from this example, the number of LOP that are formed from the ozonation of pulmonary lipids is a finite number of species that can be synthesized and tested both in vitro and in vivo for biological effects.

3. Biological effects of LOP

Airway epithelial cells respond to stimulation with the release of a variety of pro-inflammatory lipid mediators such as eicosanoids [23,24] and PAF [25]; reactive oxygen species [26,27]; and cytokines [28,29]. Synthesis and release of many

Compound Class	Lipid Precursor	Structure	Abbreviated Name
I. Aldehydes	Palmitoleic-containing Lipid	$CH_3(CH_2)_5CHO$	Ald-C7
	Oleic-containing Lipid	$CH_3(CH_2)_7CHO$	Ald-C9
	1-Palmitoyl-2-(palmitoleoyl, oleoyl or linoleoyl)-sn-glycero-3-phosphocholine	$CH_3(CH_2)_{14}CO_2CH_2$ $OHC(CH_2)_7CO_2CH$ $CH_2OP(O)_3(CH_2)_2N(CH_3)_3$	PC-Ald
II. Hydroxyhydroperoxides	Palmitoleic-containing Lipid	$CH_3(CH_2)_5CH(OH)OOH$	HHP-C7
	Oleic-containing Lipid	$CH_3(CH_2)_7CH(OH)OOH$	HHP-C9
	1-Palmitoyl-2-(palmitoleoyl, oleoyl or linoleoyl)-*sn*-glycero-3-phosphocholine	$CH_3(CH_2)_{14}CO_2CH_2$ $HOO(HO)HC(CH_2)_7CO_2CH$ $CH_2OP(O)_3(CH_2)_2N(CH_3)_3$	PC-HHP
III. Criegee Ozonide	1-Palmitoyl-2-Oleoyl-sn-glycero-3-phospho choline	$CH_3(CH_2)_{14}CO_2CH_2$ $CH_3(CH_2)_7HC(O)(O)CH(CH_2)_7CO_2CH$ $CH_2OP(O)_3(CH_2)_2N(CH_3)_3$	POPC-Oz

Fig. 3. Structures and their acronyms for LOP that are formed when various types of pulmonary lipids undergo ozonation.

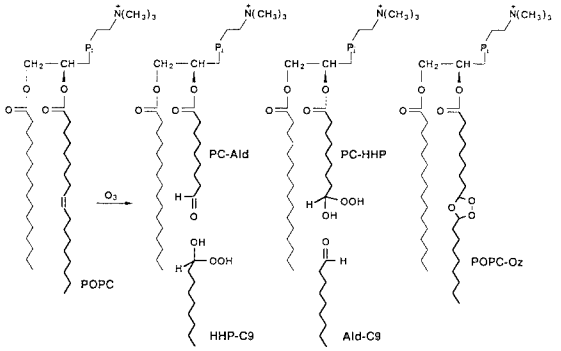


Fig. 4. The LOP formed when POPC undergoes ozonation. POPC is an important unsaturated lipid in pulmonary tissue, so the LOP shown are possible relay molecules for the toxicity of ozone into deeper tissue strata than ozone itself can penetrate.

of these substances is stimulated by ozone exposure [25]. The in vitro exposure of epithelial cells to ozone results in dose-dependent increases in PLA_2, PLC, and PLD activity [25]. The activation of PLC in epithelial cells appears to be G-protein dependent [30]. This suggests that airway epithelial cells can respond to stimuli through complicated signal transduction pathways that involve specific receptors that link G-proteins, PLA_2, PLC, and PLD. We therefore have tested whether LOP are the signal molecules that are responsible for these effects.

Our preliminary data show that individual LOP activate specific lipases both in vitro in a liposomal system [31] and in cells in culture [32]. Thus, LOP do appear to produce some of the same effects that have been observed for, and attributed to, ozone itself.

4. Cell culture data

Human bronchial epithelial (BEAS 2B) cells were incubated for 60 min with the LOP shown

Table 3
Polarized release of AA from BEAS 2B cells exposed to LOP (percent change from control) [32]

Measured effect	Ald-C7	HHP-C9	PC-Ald	POPC-Oz
Apical AA release (percent of control)	94 ± 1 P = NS	90 ± 1 P = NS	391 ± 7 $P < 0.01$	314 ± 7 $P < 0.01$

Table 4
The effects of LOP on PLC-mediated IP accumulation in BEAS 2B cells (percent change from control) [32]

LOP	PC-HHP	HHP-C9	PC-Ald	POPC-Oz
IP_1 (percent control)	145 ± 1 $P < 0.01$	139 ± 1 $P < 0.01$	74 ± 1 P = NS	73 ± 1 P = NS

in Fig. 3, at 10 μM concentration. The media from the apical compartments were collected and arachidonic acid (AA) release was measured. The results are shown in Table 3 as percent of control. There was a significant increase in [^{3}H]AA release after incubation with either the ozonide of POPC (POPC-Oz) or the derived aldehyde (PC-Ald). Compared to control and at a concentration (10 μM) that is 10-fold lower than other reported effects of similar LOP [12]. Neither the Ald-C9 nor HHP-C9 caused a significant release. Furthermore, these results are similar to those we obtained during exposure to ozone itself [33]. In that study, BEAS 2B cells were prelabelled with [^{3}H]AA and then stimulated with melittin (2 μg/ml) and [^{3}H]AA release occurred predominantly into the apical compartment. However, when the cells were exposed to ozone, the predominant [^{3}H]AA release occurred in the basolateral compartment. Both the ozone-induced and HHP-C7-induced polarized release of [^{3}H]AA appears to be due to PLA_2 activation since no PLC activation was subsequently found.

To study the effects of LOP on PLC-mediated inositol phosphate (IP) accumulation, BEAS 2B cells were incubated with [^{3}H]myo-inositol (1 μCi/ml) for 72 h and then exposed to LOP (10 μM each), using similar controls as above (see Table 4). The LOP studied included PC-Ald, HHP-C9, PC-HHP, and POPC-Oz (see Fig. 3).

These studies demonstrate that LOP have different effects on AA release (presumably due to PLA_2 activation) and IP accumulation (due to PLC activation) similar to what is found during ozone exposure [25].

5. Membrane effects of LOP

In a liposomal model membrane system, and with POPC as the lipid, we have found that PLA_2 recognizes and hydrolyzes the POPC-Oz with a rate comparable to AA [31]. This could represent a defense mechanism against ozone, since this process would clear ozonides from cell membranes. However, this process also liberates a fatty acid ozonide, which may have biological effects.

In the same system, PC-Ald is not recognized by PLA_2; however, when PC-Ald is incorporated into a liposomal membrane, the rate of hydrolysis of unaltered fatty acid PC by PLA_2 is increased. We suggest that this results from an order-disrupting effect that is produced when damaged and more hydrophilic LOP are incorporated into a membrane [31].

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Genetic susceptibility to ozone exposure

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Abstract

Because of the impact that oxidizing air pollutants such as ozone (O_3) may have on public health, identification of factors that influence susceptibility to exposure remains a critical issue. The role of *genetic background* as a susceptibility factor is becoming increasingly clear. In this paper, evidence is reviewed which suggests that susceptibility to O_3 is a heritable trait in humans. Experimental studies are also described that characterize the mode of inheritance of O_3-induced lung injury in inbred strains of mice. It is suggested that future investigations should strive to identify phenotypic markers of susceptibility as a means to identify individuals who are genetically at risk for the development of oxidant-induced lung injury.

Keywords: Inflammation; Pulmonary function; Epithelial dysfunction; Inbred mice; Human subjects; Asthma

1. Introduction

In many industrialized cities throughout the world, oxidizing air pollutants have become an important public health concern. Among these oxidants, O_3 is the most potent. Acute O_3 exposures cause decrements in pulmonary function, alter ventilation, induce inflammation, and may alter host immune defenses. It is estimated that in the United States alone, more than 50% of the population live in communities where concentrations of O_3 approach or exceed the National Ambient Air Quality Standard (NAAQS) of 0.12 parts per million (PPM), and the number of affected individuals continues to increase. The recent associations between peak O_3 levels and hospital admissions for the elderly [1] as well as exacerbation of asthma [2–5] underscore the important detrimental effects of oxidant pollutants on the lung.

Because of the potential impact that exposures to O_3 and other oxidants may have on public health, identification of the factors that determine individual susceptibility to the adverse effects of exposure is critical. The United States Clean Air Act of 1970 and the Amendments to the Act specify that the NAAQS for O_3 and other criteria pollutants was to be set low enough to protect all susceptible individuals within the population [6]. However, the determinants of susceptibility and the criteria for identification of

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a 'susceptible' individual are not clear. For discussion purposes, the determinants of susceptibility may be broadly classified as extrinsic and intrinsic (host), though it is obvious that there may be interactions or inter-dependence between these categories. Extrinsic factors may include socioeconomic status, previous exposure, and physical factors such as altitude and temperature. Intrinsic factors include age, gender, pre-existing disease, and genetic background. In the remainder of this monograph, I shall review studies that provide evidence for genetic susceptibility to various aspects of O_3 exposure. I begin by describing the inter-individual variation in pulmonary function and inflammatory responses to controlled O_3 exposure in healthy human subjects that serves as presumptive evidence of genetic predisposition to these effects. Next, I briefly describe a few studies that have examined the effect of O_3 in asthmatics, a sub-population that may be genetically predisposed to the effects of this air pollutant. I conclude with a review of animal modeling of genetic predisposition to O_3 exposure that has begun to characterize the mode of inheritance and potential mechanisms of pulmonary responses.

2. Genetic predisposition to ozone exposure in humans

2.1. Pulmonary function

The contribution of genetic background to acute O_3-induced changes in pulmonary function in humans has been largely inferred from well-characterized within-study differences in individual responsivity. For example, McDonnell and colleagues [7] found that, among healthy male subjects exposed to 0.40 ppm O_3, the change in forced expiratory volume in one second (FEV_1) ranged from 3 to 48% and the change in specific airways resistance (sR_{aw}) ranged from −20 to 80%. These investigators later demonstrated that inter-individual differences in FEV_1 were highly reproducible for as long as 10 months (when the study was completed), and they concluded that the differences were due primarily to intrinsic responsiveness to O_3 exposure [8]. Evidence of inter-individual variation in pulmonary function responses has subsequently been reproduced by other laboratories. Aris et al. [9] described distributions of pulmonary function responses (FEV_1, sR_{aw}) to 0.20 ppm O_3 in healthy subjects that were unimodal and negatively skewed, indicating that all individuals responded to O_3 but some were more responsive than others. Weinmann et al. [10] confirmed the unimodal, skewed (gamma) distribution of FEV_1 and forced vital capacity (FVC) in 64 subjects exposed to 0.35 ppm O_3. They further demonstrated that inter-individual variation in the volume-adjusted FEF_{25-75} response (a putative measure of small airways narrowing) was explained by a distribution that differed from unimodal, negatively skewed and suggested that small airways may be a significant component of the functional response to O_3. Finally, Linn et al. [11] and Schelegle et al. [12] have identified 'responders' and 'non-responders' to acute O_3 exposure in healthy volunteers based on changes in FEV_1.

2.2. Inflammation

Significant inter-individual variation in inflammatory responses to O_3 has also been demonstrated in human subjects. Aris et al. [13] described a wide range of polymorphonuclear leukocyte (PMN) counts in bronchial biopsies obtained from healthy subjects 18 h after a 4-h exposure to 0.20 ppm O_3. Analyses of lung responses to O_3 exposure by bronchoalveolar lavage (BAL) have also indicated inter-subject variability. Koren et al. [14] reported that the % PMNs recovered 18 h after exposure to 0.4 ppm O_3 ranged from 6.0 to 21.3% in 11 healthy subjects, and Seltzer et al. [15] reported a range of 1.6–35.2% PMNs recovered 3 h after a 2-h exposure to 0.4–0.6 ppm O_3 in 10 subjects. In five subjects exposed to 0.3 ppm O_3, Schelegle et al. [16] found that the % PMNs from the proximal airways lavage ranged from 8 to 24% 6 h after exposure. Similar inter-individual variation was found in PMNs, fibronectin, and interleukin (IL) 6 recovered from subjects 18 h after exposure to 0.10 or 0.08 ppm O_3 [17]. Interestingly, the

studies by Schelegle et al. and Aris et al. failed to demonstrate a correlation between the cellular inflammatory infiltration and pulmonary function responses in O_3-exposed subjects. These observations suggest that the functional and cellular responses are likely not co-dependent, and that they are regulated by different genetic mechanisms.

Collectively, these studies of normal healthy human subjects provide strong evidence that there is a heritable component to the pulmonary function and inflammatory responses to O_3, though they do not exclude the possibility that intrinsic factors such as age [18] may contribute to observed inter-individual variation. To confirm the contribution of genetic background and to begin to understand the pattern of inheritance of O_3 responsiveness, studies designed to examine O_3 effects in monozygotic and dizygotic twins [19] or aggregation of O_3 responsiveness in families are necessary steps.

3. Ozone and asthma

Sub-populations that may be genetically predisposed to O_3 include asthmatics. It is becoming increasingly clear that the pathogenesis of asthma has heritable components [20], and because asthmatics have heightened airways reactivity and lung inflammation, it has been suggested that these individuals may be at risk to the effects of O_3. Clinical studies designed to study the effects of acute O_3 exposure on airways reactivity in asthmatics have been equivocal. Only the study by Kreit et al. [21] has shown that individuals with heightened airways reactivity become more reactive than individuals with normoreactive airways after O_3 exposure. Others have been unable to demonstrate that pre-existing airway hyperreactivity is a risk factor for the development of O_3-induced hyperreactivity [22]. The discrepancy between studies has not been resolved, but it may be reasonable to suspect that it is related to variable etiologies in asthmatic subpopulations and/or genetic background (e.g. basal airways reactivity or susceptibility to O_3-induced lung inflammation and injury).

Two recent studies have suggested that asthmatics may be more susceptible to O_3-induced airways inflammation than healthy normals. Basha et al. [23] found statistically significant increases in BAL concentrations of IL-8, IL-6, and PMNs in asthmatics after acute exposure to 0.20 ppm O_3, but not in non-asthmatics after O_3. There were no O_3-induced changes in pulmonary function in either group. Similarly, McBride et al. [24] exposed asthmatics and healthy normal subjects to filtered air or 0.24 ppm O_3 and demonstrated significant increases in PMNs and epithelial cells in nasal lavage of asthmatics but found no evidence of inflammation in normal subjects. These authors also detected no O_3-induced changes in pulmonary function in either population. Both groups concluded that asthmatics were more sensitive to the acute inflammatory effects of O_3 exposure than non-asthmatics. The dissociation of O_3-induced inflammation and pulmonary function changes in asthmatics (and normal subjects; see above) may have important implications. Undetected small airways inflammation induced by oxidant exposure may lead to chronic injury and remodeling of the lung and/or enhance susceptibility to subsequent challenge with antigen, virus, or other air pollutants.

4. Animal models of genetic predisposition to ozone

Availability of inbred strains of mice (and other species) provides investigators with the tools to understand the basis of biological variability in individual responses to environmental challenges, including airborne pollutants, infectious agents, and allergens. A well-designed study with multiple strains will enable sampling of a wide range of phenotypic variation within the test species, indicate the relative contributions of genetic background and environment to the response under study, and is statistically powerful, giving fewer false positive and/or negative results. Another strength of genetic animal models is the ability to test hypotheses about mechanisms of the response using methods of

linkage analysis, and ultimately to identify the chromosomal location(s) of the gene(s) and the primary defect which leads to the disorder. Because there is considerable homology between human and mouse genomes, there is a great likelihood that genetic susceptibilities described in the mouse will be relevant to human populations.

4.1. Inflammation and epithelial dysfunction

Inter-strain variation in the inflammatory effects of O_3 exposure have been described in rats and mice. Costa et al. [25] determined that two inbred strains of rats were differentially susceptible to the edemagenic properties of acute O_3 exposure, suggesting a role for genetic background in this response. Goldstein et al. [26] demonstrated inter-strain differences in the lethal effects of O_3 exposure in inbred mice, and they suggested that O_3 susceptibility may have a genetic basis. In a series of studies with inbred mice, Kleeberger and colleagues [27–32] have investigated the genetic control of susceptibility to lung inflammation induced by acute, subacute, and chronic O_3 exposures. Initially, significant inter-strain differences in the magnitude of PMN infiltration and lung permeability were characterized after a 3-h exposure to 2 ppm O_3 [27]. Non-segregant first filial (F_1) and segregant back-cross (BX) and F_2 populations derived from differentially susceptible progenitor C57BL/6J (B6) and C3H/HeJ (C3) mice were then characterized (phenotyped) for their inflammatory responsiveness to acute O_3 exposure. The ratios of susceptible to resistant phenotypes for PMN infiltration in these populations fit the predictions for single gene (Mendelian) inheritance and the chromosomal locus *Inf* was designated with at least two alleles: Inf^s, carried by the susceptible B6 strain; Inf^r, carried by the resistant C3. Interestingly, the lung permeability response to acute O_3 exposure did not follow Mendelian inheritance patterns. To determine whether *Inf* also controlled O_3-induced changes in mouse tracheal electrophysiology, Takahashi et al. [28] measured the decrement in tracheal transepithelial potential (V_T) 6 h after acute O_3 exposure in six inbred strains as well as B6 and C3 mice and progeny derived from them. Although susceptibility to O_3-induced change in V_T appeared to be controlled by a single gene, lack of concordance between the strains for O_3-induced change in V_T and inflammation suggested that the genetic factors that determine the two responses to acute O_3 exposure were not identical.

Similar experimental procedures were followed to determine whether susceptibility to more environmentally relevant concentrations of O_3 was also controlled by the *Inf* locus. The B6 and C3 strains were also differentially susceptible to continuous 72-h (subacute) exposure to 0.3 ppm O_3, and ratios of susceptible to resistant phenotypes for PMN influx in segregant and non-segregant populations conformed to those predicted for single gene inheritance [29]. Recombinant inbred (RI) strains of mice derived from B6 and C3 progenitors were also phenotyped for susceptibility to acute and subacute exposures and the strain distribution patterns (SDP) were compared for concordance. The presence of discordant cross-over phenotypes (i.e. those RIs that were susceptible to one exposure and resistant to the other) demonstrated that different genes regulated susceptibilities to the two exposures. Consequently, the *Inf-2* locus was designated for susceptibility to sub-acute O_3 exposures [30]. Further, some of the RIs were *more susceptible* (i.e. hypersusceptible) to acute and sub-acute O_3 exposures than the B6 progenitor [30]. The presence of another distinct phenotype in the RI set indicated that another minor modifying gene(s) contributed to the inflammatory response to each exposure. We are currently attempting to determine whether *Inf* or *Inf-2* regulate inflammatory and epithelial cell responses to chronic exposures to O_3, or whether other genes control susceptibility. B6 and C3 mice and their progeny have been exposed to 0.30 ppm for 8 h, five times/week, for up to 6 months; between 8-h exposures, mice were exposed to background 0.06 ppm O_3. As with acute and sub-acute exposures, B6 mice had significantly greater lung injury than C3 mice after chronic O_3 exposure [31]. Preliminary re-

sults suggest that susceptibility to lung injury induced by chronic O_3 exposure is a quantitative trait in these mice [31].

4.2. Airway reactivity

As O_3 is also known to induce airways hyperreactivity in human subjects [15], individuals with chronic inflammation and hyperreactive airways are considered to be at risk to the effects of oxidant exposures [23,24]. To establish whether inherent airways hyperreactivity is a risk factor for the development of O_3-induced lung injury, Zhang et al. [32] measured the change in airways reactivity following acute O_3 exposure in seven inbred strains of mice with genetically-determined differences in basal reactivity to acetylcholine (ACh). These investigators found that, while some strains of mice that were base-line hyperreactive to ACh also developed O_3-induced hyperreactivity, others were not affected. Similarly, among base-line hyporeactive strains, some developed O_3-induced hyperreactivity while others did not. Interestingly, there was complete concordance between O_3-induced hyperreactivity and susceptibility to O_3-induced airways injury, suggesting that there may be common mechanisms that contribute to the pathogenesis of these events.

These studies have demonstrated the utility of inbred strains and genetic models for the analysis of pathophysiologic processes in the lung, such as those induced by air pollutants. The next step in the development of these models is to determine precise chromosomal locations of *Inf*, *Inf-2*, and other associated loci. Identification of chromosomal map assignment(s) affords the search for a candidate gene that accounts for the genetic control of O_3 susceptibility in the mouse and human. One of the goals of these studies is to determine linkage associations of susceptibility to the effects of O_3 exposure with easily identified phenotypic markers that are homologous in the mouse and human. A phenotypic marker would thus provide a means to identify individuals who are at risk to the development of oxidant-induced lung injury.

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Long-term toxicity studies of ozone in F344/N rats and B6C3F1 mice

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Abstract

The toxicity and carcinogenicity of ozone was evaluated in Fischer 344/N rats and B6C3F1 mice exposed to 0, 0.12 (2 years only), 0.5 or 1.0 ppm ozone by inhalation for 2-year and lifetime exposures. A 2-year cocarcinogenicity study (male rats only) included the subcutaneous administration of 0, 0.1 or 1.0 mg/kg/body wt. of 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) for the first 20 weeks along with inhalation exposure to 0 or 0.5 ppm ozone followed by additional 84 weeks of ozone exposure alone. Ozone exposure in rats did not cause an increased incidence of lung neoplasms. In the cocarcinogenicity study, ozone exposure did not have an additive carcinogenic effect. Lifetime and 2-year ozone exposure was associated with a marginal increase in lung tumors in male B6C3F1 mice and a more pronounced increase in females. Unique mutations in the K-*ras* gene were found in the mouse lung neoplasms from the ozone-exposed mice.

Keywords: Ozone; Toxicity; Carcinogenicity; Bioassay; Lung; Neoplasia

1. Introduction

Ozone remains one of the 3 most important air pollutants worldwide [1], yet the potential long-term toxicity and carcinogenicity in rodents is not clear. Ozone has been found to either enhance or inhibit the development of pulmonary neoplasms in the mouse [2]. Ozone is mutagenic [3] and has carcinogenic potential in rodents, since it causes DNA damage caused by oxidative stress from hydroxyl radicals, superoxide, singlet oxygen, and hydrogen peroxide. The State of California and the Health Effects Institute (HEI) nominated ozone to the National Toxicology Program (NTP) for long-term studies in rats and mice to determine the potential toxicity and carcinogenicity of long-term ozone exposure. The studies reported here include exposures at the current EPA standard (0.12 ppm), the maximum concentration considered to be compatible with long-term survival (1.0 ppm) and an intermediate concentration (0.5 ppm). A second ozone inhalation study was included in which 0.5- and 1.0-ppm exposures were continued to 30 months based on lifetime studies in rats in which the majority of lung tumors developed after 24 months [4]. A third study was included in which male rats were administered 2 concentrations of

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a known pulmonary carcinogen, 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) [5–7], and exposed to 0.5 ppm ozone to determine the cocarcinogenic potential of ozone.

2. Materials and methods

Groups of 50 male and 50 female Fischer 344/N (F344/N) rats and B6C3F1 mice were exposed to 0, 0.12, 0.5 and 1.0 ppm ozone 6 h/day, 5 days/week for 2-year and 0, 0.5 and 1.0 ppm ozone for lifetime studies. In the cocarcinogenicity study, groups of 50 male rats were injected s.c. with 0, 0.1 and 1.0 mg/kg NNK (greater than 99% purity) in trioctanoin (0.1 ml) 3 times/week followed by ozone exposure (0 or 0.5 ppm) 6 h/day, 5 days/week for 20 weeks; thereafter to ozone exposure alone (0 or 0.5 ppm) for 84 weeks.

Male F344/N rats and B6C3F1 mice were obtained from Simonsen Laboratories (Gilroy, CA) at 4–5 weeks of age. Animals were randomly assigned to ozone exposure or control groups after a 10–14-day quarantine period. Animals were housed in modified Hazelton 2000 inhalation chambers. The lifetime studies in the rats were terminated at 124 weeks with 1 male and approximately 7 females per group remaining, whereas the lifetime mouse studies were terminated at 130 weeks when there was approximately 20% survivors per group. The rodents had access to NIH 07 rodent chow (Ziegler Brothers) when not in the chamber and water was available ad libitum.

Exposure conditions have been reported in detail [8–10]. Chamber air was filtered and conditioned prior to introduction to the chamber. Ozone was generated by corona discharge using an OREC model 03V5-0 ozonator and 100% oxygen. There were 8 ports per chamber and each port was sampled approximately twice per h. The percent of samples within the concentration range was 89–99% for the various chambers, and the mean chamber concentration for all chambers were generally within 1% of the target range. The ozone concentrations in the control chamber were less than 0.002 ppm (below level of detection), 0.12 ± 0.01, 0.51 ± 0.02, and 1.01 ± 0.05 for the 2-year rat studies and 0.50 ± 0.03 and 1.01 ± 0.06 for the lifetime mouse studies.

All animals found moribund or dead and those killed at terminal sacrifice were subjected to a complete necropsy. Approximately 43 tissues and all gross lesions were fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological examination. The nasal passage was sectioned at (1) the level of the incisor teeth, (2) midway between the incisors and the first molar and (3) the middle of the second molar for examination. Sections of 3 levels of larynx were prepared and examined following the method of Renne et al. [11] and included the base of the epiglottis, ventral pouches, and vocal processes. An entire coronal (perpendicular to the saggital plane and parallel to the long axis of the body) section of the right lobes and the left lung lobe were prepared for examination. Lung lesions were diagnosed using standard morphologic criteria [12].

Formalin fixed paraffin-embedded lung neoplasms greater than 1 mm diameter were evaluated by polymerase chain reaction (PCR)-based assays, including single stranded conformation polymorphism (SSCP) and direct sequencing analysis. Lung tumors from 9 controls and 26 female ozone-exposed mice were examined. DNA from the formalin-fixed paraffin-embedded neoplasms was isolated. Using SSCP, the DNA was screened for K-*ras* mutations at exon 1 and exon 2. The mutations at codon 12, 13 and 61 were identified by direct sequencing analysis [13].

3. Results

Air flow had to be increased 5-fold from 12 to 60 air changes per h to achieve chamber uniformity. At this increased airflow, the maximum between – and within – port variability was within 5% for all studies. Additional details on chamber concentrations are provided in the NTP Technical Report [10].

Ozone had no effect on survival in either male or female rats, or male and female mice, in either the 2-year or lifetime studies [10]. The mean

body weights of the 0.12- and 0.5-ppm male and female rats and mice were similar to those of the controls throughout the studies, whereas the mean body weights of males and females of both the F344/N rats and B6C3F1 mice in the 1.0-ppm group were slightly lower than those in controls in both the lifetime and 2-year studies [10].

The incidences of pulmonary neoplasms observed in the rat lung are shown in Table 1. All lesions were solitary except in 1 male exposed to 1.0 ppm ozone for 2 years which had multiple alveolar/bronchiolar adenomas. Adenomas were papillary to glandular, usually with a uniform growth pattern that obliterated the underlying alveolar architecture. Carcinomas were more nodular, occasionally invasive and had more pleomorphic growth patterns. The incidence of neoplasms did not increase significantly with ozone exposure and those occurring in ozone-exposed rats were morphologically similar to those in controls.

In the cocarcinogenicity study, there was an increased incidence of focal alveolar hyperplasia with marked cellular atypia, and alveolar/bronchiolar adenoma or carcinoma (combined) in rats administered 1.0 mg/kg NNK with or without exposure to ozone (data not shown). Inhalation of ozone did not affect the incidence of atypical hyperplasia or the incidence of pulmonary tumors in rats administered NNK [10].

In mice, the incidence of pulmonary neoplasms increased with increasing ozone exposure (Table 2). In the 2-year study, the most prominent increased incidence was alveolar/bronchiolar

Table 1
Neoplastic lesions of rat lung with 2-year and lifetime ozone exposure

	Male				Female			
Conc. (ppm)	0	0.12	0.5	1.0	0	0.12	0.5	1.0
2-year studies ($N = 50$)								
Al/Br adenoma	1	2	2	3	0	0	2	0
Al/Br carcinoma	1	1	1	1	0	0	0	0
Lifetime studies ($N = 50$)	0	–[a]	0.5	1.0	0	–[a]	0.5	1.0
Al/Br adenoma	2	–	3	0	0	–	1	1
Al/Br carcinoma	0	–	1	0	1	–	1	0

[a] There was no exposure group at 0.12 ppm for the lifetime study.
Al/Br, alveolar/bronchiolar. The number shows the number of rats with each tumor per exposure group.

Table 2
Neoplastic lesions of mouse lung with 2-year and lifetime ozone exposure

	Male				Female			
Conc. (ppm)	0	0.12	0.5	1.0	0	0.12	0.5[a]	1.0
2-year studies ($N = 50$)								
Al/Br. adenoma	6	9	12	11	4	5	5	8
Al/Br. carcinoma	8	4	8	10	2	2	5	8
Adenoma/carcinoma[b]	14	13	18	19	6	7	9	16
Lifetime studies ($N = 50$)	0[a]	–[c]	0.5[a]	1.0	0	–[c]	0.5[a]	1.0
($N = 50$)								
Al/Br. adenoma	8	–	8	9	3	–	3	11
Al/Br. carcinoma	8	–	15	18	3	–	5	2
Adenoma/carcinoma[b]	16	–	22	21	6	–	8	12

[a] Only 49 mice/group.
[b] Combined tumor incidence of tumor-bearing animals.
[c] There was no exposure group at 0.12 ppm for the lifetime study.
Al/Br, alveolar/bronchiolar. The number shows the number of mice with each tumor per exposure group.

carcinomas in female mice. The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) occurred with a significant positive trend and the incidence in the 1.0-ppm females at 2 years was significantly increased. The incidence in this group (16/50, 32%) exceeded historical controls (58/659, 9%). In the lifetime study, increased incidences of alveolar/bronchiolar adenoma or carcinoma (combined) occurred in exposed groups of male and female mice (Table 2); the incidence of adenomas in the 1.0-ppm ozone group females was significantly greater than controls. In male mice there was an increased multiplicity of tumors (controls, 2; 0.5 ppm, 5; 1.0 ppm, 4). When the incidence of neoplasms in the 2-year and lifetime studies are considered together, the significance of the combined alveolar/bronchiolar neoplasms increases [10].

Analysis for genetic alterations in the K-*ras* gene in the B6C3F1 mice revealed mutations in codon 12, 13, and 61 in lung neoplasms from both control and ozone-exposed female mice [13]. Mutations in the K-*ras* gene were found in 3/9 (33%) lung neoplasms from control and 19/26 (73%) lung neoplasms of the ozone-exposed animals. Several mutations appeared in the ozone-exposed animals; 10 tumors contained activated K-*ras* at codon 12 including GGT → GTT, GGT → GAT and GGT → TGT; 1 tumor at codon 13 GGC → GAC; and 8 tumors at codon 61 CAA → CTA. In the ozone control mice the mutations were only at codon 12 and 13. In control mouse lung neoplasms from other studies at our institute, 7/66 neoplasms contained activated K-*ras* with mutations at codon 61; however none had CTA mutations. There was no association between the presence of activated K-*ras* or specific mutations and the size or morphology of the lung neoplasms.

4. Discussion

This purpose of this study was to determine whether long-term ozone exposure would increase cancer incidences in rats or mice. We also evaluated whether ozone could act as a cocarcinogen. Ozone is the major component in photochemical smog, and there was a lack of long-term animal studies to evaluate this potential human hazard. Our rodent studies included 2-year and lifetime studies in rats and mice and a cocarcinogenicity study in male rats. The 4–6% lower body weight in the 1.0-ppm exposure groups for both rats and mice, and the nearly 100% incidence of pulmonary toxicity lesions suggest that a maximum concentration was reached in these studies. Ozone concentrations of 2.0–4.0 ppm are lethal to rodents or cause significant pulmonary edema [14], and thus concentrations greater than 1.0 ppm may not have been tolerated. The lifetime study was terminated at 123 weeks in rats, when all male rats except 1 had died, and only a few female rats remained. In the lifetime mouse study, the termination was at 130 weeks with about 20% survival in each group. The toxicity data from this study suggests that in these rodent strains neither greater ozone concentrations nor longer exposure periods are feasible.

There have been few studies that examined the potential carcinogenicity of ozone. In a 13-month study, Ichinose and Sagai [15] reported no increase in lung neoplasms in Wistar rats exposed to 0.05 ppm ozone. In the current rat study, there was no evidence of an increase in pulmonary or nasal neoplasms in either male or female rats exposed to ozone for 2 years or for the life of the animal. There was also no evidence that ozone enhanced a known pulmonary carcinogen, NNK, suggesting that there is no evidence of carcinogenic activity of ozone in male or female F344/N rats exposed to 0.12, 0.5 or 1.0 ppm ozone in lifetime exposures.

The data from mice studies are less clear [2]. Hassett et al. [16] reported a slight increase in pulmonary adenomas seen grossly in a limited number of A/J mice following exposure to 0.31 and 0.5 ppm ozone alone. Swiss Webster mice exposed to 0.4 and 0.8 ppm ozone for 18 weeks had no increase in pulmonary neoplasms [17]. However, we observed an increase in pulmonary neoplasms in B6C3F1 mice in both 2-year and 130-week studies [10]. In humans, there is no conclusive link between ozone exposure and pulmonary cancer [18]. In a cocarcinogenicity

study in mice, Hassett et al. [16] reported no increase in pulmonary adenomas in A/J mice treated with urethane followed by exposure to 0.31 and 0.5 ppm ozone.

While the rat studies were negative for carcinogenicity, the mice consistently showed an increase in lung neoplasms, but in many cases, the results were marginal. For example, in the male mice in the 2-year study, the combined alveolar/bronchiolar adenoma or carcinoma incidence was significantly increased, while in the lifetime study the incidence was increased but not significantly. In general, with longer exposure to a carcinogenic agent, the effect is more pronounced. In female mice, the same effect as in males, a significant increase in pulmonary cancer at 2 years but not with lifetime exposure was seen. In support of a carcinogenic effect, the multiplicity of neoplasms was increased, but only for male mice in both studies. When the incidence of lung neoplasms in both the 2-year and lifetime studies was combined, there was a significant increase in alveolar/bronchiolar adenoma or carcinoma for both male and female mice. The increasing trend for neoplasms in both male and female mice and in both the 2-year and lifetime studies are consistent with ozone exposure enhancing the incidence of pulmonary neoplasms in mice.

It is important to determine whether ozone is inducing unique lesions or simply promoting the spontaneous lung neoplasms. Mutation spectra in codon 12, 13 and 61 of the K-*ras* gene in ozone-induced neoplasms were similar to those of spontaneous lung neoplasms [19]. A higher frequency of K-*ras* mutations, however, were observed in the neoplasms from ozone-exposed mice (73%) as compared to lung neoplasms from this study controls (33%) or spontaneous neoplasms from several studies at our institute (32%). The unique A→T transversions in the second base of codon 61 accounted for 42% of the point mutations in the lung tumors. We have not seen this mutation in spontaneous lung tumors in the B6C3F1 mouse. This suggests that the CTA mutational defects in the lung tumors are most likely related to ozone exposure. Ozone remains an important air pollutant worldwide [1], and it is important to understand the molecular events that underlie the pathology of ozone toxicity. These studies showing increased carcinogenic activity of ozone in B6C3F1 mice with unique molecular lesions suggest that the potential carcinogenicity of ozone deserves further study.

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Ozone-induced human respiratory dysfunction and disease[1]

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Abstract

Exercising volunteers exposed in chambers to as little as 80 ppb O_3 for several hours exhibit impaired lung function and irritative lower airway symptoms. Comparable changes occur among children and young adults exposed to summer smog containing O_3. Intensity of the response is reproducible but varies widely among individuals. The (reversible) decrements in vital capacity are due to involuntary inhibition of deep inspiration probably mediated by nociceptive bronchial C-fibers that may be stimulated by local prostaglandin release, and can be modulated by appropriate pharmacologic agents. A second characteristic response to low O_3 levels is mucosal neutrophilic inflammation probably mediated by phospholipid-derived products and by epithelial cell-derived chemokines and cytokines, but poorly correlated with lung function changes. Fluctuations in ambient O_3 levels are associated with acute respiratory health effects in exposed populations but concomitant acid aerosol pollution is an important confounder. Whether irreversible impairment of lung function occurs among residents of chronically high ozone-pollution areas is debated.

Keywords: Ozone; Lung inflammation; Bronchial C-fibers; Lung function; Respiratory health effects

Ozone (O_3) is a secondary air pollutant, being formed in the troposphere from primary precursor pollutants (e.g. in motor vehicle engine exhaust) such as NO_x and hydrocarbons. In the presence of light NO_2 is cleaved to $NO\cdot + O\cdot$ and allows formation of O_3 ($O_2 + O\cdot$). By reconversion of $NO\cdot$ to NO_2 in complex reactions involving the hydrocarbons, and photochemical recycling of the NO_2, O_3 accumulates in ambient air and may reach levels as high as 0.3–0.4 ppm in an urban basin with heavy vehicular traffic and adequate sunlight. In the USA, Los Angeles is the prototypical area for O_3 pollution and its residents had complained of respiratory and ocular irritation during summer smog episodes for years prior to the public pressure and congressional hearings that led to enactment of the Clear Air Act.

Consistent with this atmospheric precursor-product relationship, ozone accumulation in Los Angeles ambient air appeared to lag the morning rush hour traffic by a couple of hours, thus

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producing a midday concentration peak. This observation led to design of experimental O_3 exposure protocols that were of relatively short duration and often to levels of 0.3–0.4 ppm. It has become clear more recently, however, that atmospheric O_3 transport to periurban areas occurs and that ambient summertime ozone levels may remain elevated for many hours over large and populous areas such as the northeastern USA. Thus, more recent studies have used multi-hour exposures to relatively low levels of O_3 (0.08–0.16 ppm) in an effort to mimic these exposure conditions. In all cases, the use of increased ventilation (exercise) allows demonstration of effects at much lower O_3 levels than would otherwise be the case.

Ozone, of course, is a gas and does not impair visibility. Its presence in ambient air, however, is almost invariably associated with particulate air pollution, some of which is directly related to the presence of ozone i.e. oxidative generation of aerosols of sulfate and nitrate salts from SO_2 and NO_2, respectively. The sulfate may include H_2SO_4, bisulfates, or fully neutralized salts. Thus the health effects of exposure to O_3-containing smog may not be uniquely attributable to the O_3 component, and this has emerged as a problem in the interpretation of studies of respiratory health of populations residing in regions with episodes of summertime oxidant smog.

With this prelude, let us return to controlled experimental exposures of volunteers to O_3 that demonstrate a variety of acute and subacute reversible effects on the respiratory tract. From such exposures, one can calculate that the minimum dose of O_3 required to produce detectable effects on the respiratory system is of the order of 0.5 mg (10 μmol). Considering the large surface of distribution, we see that O_3 is a potent toxicant and that its effects may involve amplification mechanisms.

In human ozone toxicology[2], the 'classical' effect of exposure of healthy volunteers is a loss of ability to take in a deep breath, accompanied by symptoms of substernal (tracheal) irritation and cough which are enhanced by attempted deep inspiration. This finding is quantified as a decrease in forced vital capacity (FVC) and in the forced expiratory volume in 1 s (FEV_1). After cessation of exposure, the signs and symptoms regress with a half-time of 1–2 h. The lung function of subjects with severe acute responses, however, may still be slightly below baseline at 24 h.

Individual responses to a given exposure protocol vary widely, but there are few known characteristics that predict responsiveness [1]. Vigorous responders are more prevalent among non-smoking adolescents and young adults than among older individuals, but no long-term longitudinal studies of the effect of aging are available. However, once an individual subject's degree of responsiveness to a particular exposure has been characterized, it is highly reproducible (at least in the short term) [2], and individual exposure concentration-response curves are also typically monotonic. This suggests a possible role for genetic factors which is currently under study in monozygotic and dizygotic twins. As discussed below, these responses can be blunted or even abolished by certain pharmacologic agents and by consecutive daily exposures.

The O_3-induced decrease in vital capacity is almost entirely due to decreased inspiratory capacity. This is not attributable either to grossly increased lung elastic recoil or to inspiratory muscle weakness, and we have proposed neurally-mediated involuntary inhibition of inspiration as the mechanism [3].

Recent studies of canine bronchial C-fibers in the vagus nerve [4,5] have demonstrated rapid onset and persistently increased firing rate of these neurons during exposure to 2–3 ppm O_3. Bronchial C-fibers are nociceptive unmyelinated sensory fibers that in addition to their central inputs also subserve local motor functions. Tachykinin peptides such as substance P (SP) are released from the nerve endings when stimulated and the arborized nerve functions antidromically to widen the field of the effects and the variety of responding cells and structures in the airway wall (mediated by neurokinin receptors). Hazbun et

[2] In this paper no consideration will be given to ozone-induced changes in DNA, mutagenesis, genotoxicity or carcinogenicity.

al. [6] have reported increased SP levels in proximal lower airways surface liquid following O_3 exposure of volunteers.

Bronchial C-fibers are stimulated by a variety of mediators such as prostaglandin E_2 (PGE_2) and bradykinin (BK). Since PGE_2 is known to be greatly increased in airways surface liquid (sampled by bronchoscopy) after O_3 exposure [7], this mediator could participate in bronchial C-fiber activation. Indeed, pretreatment with pharmacologic inhibitors of cyclooxygenase causes significant blunting of the O_3-induced decrements in lung function [8,9].

Recently, we have studied the effect of i.v. injection of the potent opioid receptor agonist, sufentanyl, immediately after an ozone exposure of normal volunteers that produced substantial decrements of lung function. We observed a rapid return of FVC and FEV_1 almost to baseline accompanied by relief of symptoms. (The injection of saline placebo did not alter O_3-induced spirometric decrements.) We also studied subjects who had little or no response to ozone. Injection of the opioid receptor antagonist, naloxone, in such subjects failed to provoke appearance of symptoms or of spirometric decrements. (Saline placebo was likewise without effect.) These findings support the hypothesis that the spirometric decrements and airway symptoms produced by O_3 inhalation are mediated by nociceptive sensory neural pathways whose function is down-regulated by opioid agonists. Endogenous endorphins, however, do not appear to be modulating responses to O_3 exposure.

In addition to impaired inspiratory capacity other characteristic O_3 exposure effects (also seen in experimental animals) include decreased tidal volume (V_T) and increased respiratory frequency (f_R). The O_3-induced alteration in respiratory pattern is readily demonstrable during exercise in humans and correlates with the severity of the lung function decrement. This, too, may be mediated by bronchial C-fibers (polymodal nociceptors), but also by irritant receptors – either stimulated 'directly' by products of O_3 metabolism, or indirectly by local tachykinin release. A recent abstract [10] reports that rats treated neonatally with capsaicin, so as to chemically ablate the C-fiber system, failed to develop $\downarrow V_T$ or $\uparrow f_R$ during subsequent O_3 exposure.

Decrements in lung function also occur in spontaneous exposures to ambient O_3, among children playing actively in summer camps [11] and in adult joggers [12] (even at levels lower than 0.12 ppm), and in competitive cyclists [13]. The observed decrements in lung function seem to be consistent with the ozone concentration-response relations established in the controlled exposure studies.

These respiratory effects have been interpreted as adverse health effects and have been an important element in the health risk assessment evaluation used by U.S. EPA to support a primary national ambient air quality standard (NAAQS) of 0.12 ppm O_3 (1-h average) which may be exceeded only 1 day per year. Clearly the existing NAAQS level for ozone provides little 'margin of safety' for young adults and children in terms of the acute effects just discussed. Whether these effects are sufficiently severe, however, to justify limiting exceedances to one-time-per-year could be debated.

Because prolonged exposures (with exercise-induced increased ventilation) to as little as 0.08 ppm O_3 cause decreased lung function, and because summertime ambient air O_3 levels commonly fluctuate within the 0.08–0.12 ppm range for many hours, there has been sentiment in favor of an additional ozone NAAQS at a concentration lower than 0.12 ppm, but averaged over a multi-hour time period.

A surprising feature of the acute response to ozone is that there is relatively little bronchoconstriction of the larger airways as judged by changes in airflow resistance (R_{aw} or SR_{aw}), despite a modest post-O_3 increase in bronchial reactivity to drugs like histamine or methacholine. The increases in SR_{aw} that do occur after O_3 exposure correlate poorly with spirometric decrements [14]. They can be prevented by atropine pretreatment (suggesting a reflex cholinergic motor effect on airways smooth muscle), or reversed by beta-adrenergic agonist inhalation, neither of which affect the ozone-induced

decrement of vital capacity [15]. Even asthmatic subjects do not develop exceptional bronchoconstriction during exposure to ozone (in marked contrast to their sensitivity to brief SO_2 exposure).

Nevertheless, there is reason to suspect the existence of ozone effects on small airways caliber: (1) FEV_1 decrements often exceed FVC decrements – a finding which should not occur simply due to a decrease of inspiratory capacity; (2) Iso-volume maximum expiratory flow appears to be decreased, and these changes are reported to persist for many hours despite post-exposure return of FVC and FEV_1 toward (or to) baseline [16]; (3) There appears to be a very small (but significant) increase in residual volume (RV) which suggests premature closure of small airways near end-expiration [3]; (4) Other more complex physiologic tests also suggest mild disturbance in distribution of ventilation [17]; (5) Small airways are predicted by dosimetric models to receive the highest dose of O_3 per cm^2 surface [18], and are a vulnerable zone in animals acutely or chronically exposed to ozone.

Impairment of small airways function is of particular concern since inflammation and remodeling in this area can produce chronic obstructive lung disease. This concern has been enhanced by the results of investigative bronchoscopy applied to ozone-exposed volunteers.

Bronchoscopy-associated procedures such as bronchoalveolar lavage (BAL) and airways mucosal biopsy have established that exposures to ozone identical to those that provoke acute lung function decrements cause neutrophilic (PMN) inflammation of the submucosa and surfaces of the airways [19–22]. As with the lung function decrements, there is considerable inter-individual variability in the inflammatory response to ozone. The two types of effects, however, are not positively correlated within individuals. Indeed, they may reflect events occurring in different regions (large airways vs. more distal airways) and involve different mechanisms.

The time course of the inflammatory response is more complex than that of the spirometric response, and has not been as well defined [7,23]. Neutrophils appear by 1 h after termination of exposure, may peak at ~6 h and persist for at least 18 h. Clearly there must be early pro-inflammatory events to provide the chemokine gradients and mediators that direct the adhesion of neutrophils to the airways microvasculature and their subsequent migration into and across the epithelium onto the airways surface where they are accessible to lavage. In vitro studies of cultured cell models of airways epithelium indicate that sublethal levels of ozone exposure upregulate cellular production and export of neutrophil attractants such as alpha-chemokines (IL-8, GRO) and platelet activating factor (PAF) [24,25]. In vivo biopsy studies show upregulation of ICAM-1 expression on the endothelial cells of bronchial submucosal capillaries (R. Aris, personal communication). Increased neutrophil adherence to cultured human bronchial epithelial-derived cell lines following O_3 exposure has been demonstrated, and (in part) involves interaction of epithelial ICAM-1 with the CD11b/CD18 (β_2 integrin) complex on the neutrophil surface (W. Reed, personal communication).

Ozone exposure of cells in culture causes release of arachidonate from cell membrane phospholipids. Both increased phospholipase A_2 activity and impairment of enzymatic reincorporation of arachidonate into lyso-phospholipid appear to be involved [26]. During exposure, free arachidonate may be directly attacked by ozone to form a variety of shorter chain aldehydes that have pro-inflammatory activity [27]. Likewise, the bound sn-2 arachidonyl residue of intact phospholipids of the sn-1 alkyl type may be attacked by O_3 to form shorter chain sn-2 acyl derivatives that mimic PAF [28].

Increased arachidonate metabolism in cultured cells persists after O_3 exposure [29]. Increased PGE_2 and TxB_2 are demonstrable by immunoassay in human BAL fluid obtained 1–2 h after O_3 exposure and these levels decline with time [7]. The formation of these products in O_3-exposed subjects is inhibited by pretreatment with the cyclooxygenase inhibitor, ibuprofen, but this failed to reduce the degree of neutrophilia in bronchoalveolar lavage although the lung function decrements induced by the O_3 exposure were significantly blunted [9].

The upregulation of alpha-chemokine (Cys-X-

Cys structural motif) production by O_3 exposure suggests that, as has been described for H_2O_2 and other oxidants [30], this form of oxidant stress may activate NF-κB and perhaps other transcriptional regulators that enter the nucleus and cooperate to enhance transcription of genes with upstream NF-κB response elements [31]. These include alpha (but not beta) chemokines as well as IL-6. High levels of IL-6 are found in BAL fluid soon after O_3 exposure and the levels decline with time [7]. Viable airway epithelial cells are probably the principal source of these chemokines, cytokines and eicosanoids.

Whether, and under what circumstances, this acute neutrophilic airways inflammation is capable of damaging the airways epithelium (or other elements) is not established. Ozone exposure impairs the epithelial permeability barrier to passive solute diffusion [32], but in animals the role of neutrophils in this phenomenon is both affirmed [33] and denied [34]. Indeed it has been suggested that the neutrophils may support healing of ozone-injured epithelium. The epithelium is capable of secreting cytokines (e.g. endothelin-1) which could support mitogenic activity in airway wall mesenchymal elements, and thus lead to remodelling. Whether such events might result from acute or repeated O_3 exposure is not yet clear.

Morphologic studies have shown that ozone exposure also directly damages surface cells. Ciliated cells, the predominant cell type in the airways epithelium, appear to be especially vulnerable to direct O_3-induced deciliation and to lethal injury, but it is unknown whether the latter represents activation of programmed cell death (apoptosis). Dead cells are extruded from the epithelium and are replaced by division (DNA synthesis) and differentiation of progenitor cells.

Given this picture of O_3-induced acute inflammation in normal volunteers, one might expect individuals with preexisting diffuse airways diseases like asthma or chronic obstructive lung disease (COLD) to prove especially vulnerable. Indeed, increased levels of summertime ambient air oxidant pollution (particulate acids and salts as well as ozone) are associated with increased hospital admissions for respiratory (but not other systems) diagnoses [35,36]. An association between fluctuations in ambient air ozone and daily mortality is suggested by the analyses of Kinney and Özkaynak [37,38].

Controlled exposures of COLD patients to ozone have, however, failed to reveal exceptional sensitivity – at least to the spirometric effects [39,40]. Some of the subjects were active smokers (which reduces acute responsivity to ozone), they were all relatively older (again reducing sensitivity), and were not capable of more than modest exercise (minute ventilation of <30 l). Bronchoscopic studies have not been performed in this type of patient after O_3 exposure. A trend toward a very small reduction of arterial O_2 saturation (pulse oximetry) has been observed. If present, it might imply impairment of ventilation/perfusion relations and possibly of small airways function.

As noted previously, mildly affected asthmatic patients appear not to be exceptionally reactive to controlled O_3 exposure in terms of acute effects on lung function, and particularly bronchoconstriction. Their inflammatory response has only recently begun to be explored, focussing on allergic patients.

Using the nasal mucosa as a surrogate for the lower airways, Bascom et al. [41] and Peden et al. [42] have shown that a substantial cellular response in nasal lavage liquid was evoked by O_3 exposure of allergic rhinitics and asthmatics, respectively. Unlike normal subjects in whom similar procedures evoke only neutrophils, nasal lavage from the allergic individuals also had increased numbers of eosinophils or of eosinophil markers (eosinophil cationic protein). Markers of mast cell degranulation, however, were not increased despite a previous report of increased tryptase in nasal lavage following O_3 exposure of normal volunteers [43]. Lavage of the lower airways following O_3 exposure of allergic asthmatics revealed the expected increase of neutrophils but (surprisingly) not of eosinophils [44]. A significant role of mucosal mast cells in enhancing ozone-induced neutrophilic inflammation is suggested by studies in genetically mast-cell deficient mice [45]. Asthmatics have increased numbers of submucosal airways mast cells and might therefore exhibit increased neutrophilic inflammation after O_3

exposure. If ozone-induced inflammation is indeed mediated largely by airways epithelium-derived factors, then products secreted by submucosal and intraepithelial mast cells may modulate epithelial cell responses to ozone in a paracrine fashion.

It is possible that the effects of O_3 exposure interact with those of other inhaled agents to produce significant health effects. Simultaneous exposure to O_3 and acid aerosol (to mimic smog) seems to somewhat enhance the spirometric effects, but not to a statistically significant degree. Other protocols have examined sequential exposures to ozone and various air pollutants.

Of particular interest at present is the interaction between O_3 and specific antigen in allergic asthmatics. Some (but not all) reports point toward an increased response when ozone exposure is followed by either nasal [42] or inhaled [46] antigen challenge. Jörres et al. [47] showed a mean 3.3-fold reduction of the dose of inhaled specific antigen required to acutely decrease FEV_1 by 20% in mild asthmatics following 3-h mouthpiece exposure to 0.25 ppm O_3 with intermittent mild exercise. Of interest would be studies of O_3 exposure effects in allergic subjects previously challenged with antigen so as to increase the intensity of asthmatic airways inflammation.

Although this discussion has focussed on airways inflammation, significant alterations in function of alveolar macrophages obtained by lung lavage in ozone-exposed humans [21,24] and rodents [48,49] have also been demonstrated. In mice, this leads to sharply increased mortality due to pneumonia following challenge with inhaled aerosolized suspensions of bacteria [50].

Finally, we come to the effect of repeated exposures to O_3. Consecutive daily controlled exposures of initially responsive healthy volunteers clearly lead to abolition (by day 4 or 5) of spirometric responses. This phenomenon is often referred to 'adaptation'. It regresses (i.e. responsiveness to O_3 returns) over 4–7 days of non-exposure [51].

The effect of consecutive daily O_3 exposures on the inflammatory response as revealed by bronchoalveolar lavage has only recently been investigated in human subjects [52]. This study showed only 2% neutrophilia in post-O_3 BAL on the 5th day of consecutive daily 2-h exposures to 0.4 ppm O_3. A mean value of about 10% was anticipated for a single such exposure (historical data). Levels of other BAL fluid markers of O_3-induced inflammation (e.g. IL-6, PGE_2) were also reduced, but not a marker of cell injury (LDH). Biopsies were not done and it is conceivable that tissue neutrophilia was present, but that the neutrophils failed to cross the epithelium onto the airways surface. Recovery of acute O_3 responsiveness was found 10 and 20 days later, but may not have been complete.

Although chronic animal exposure studies abound, there are surprisingly few data specifically examining the subacute (several days) effect of repeated daily exposures to ozone on parameters reflecting lung inflammation, including mediators and cells in BAL. Donaldson et al. [53] showed a significant decrease of BAL neutrophilia over a 4-day period in rats exposed to 0.6 and 0.8 ppm O_3 for 7 h per day on consecutive days. Van Bree et al. [54] showed marked attenuation of neutrophil, albumin, fibronectin, and IL-6 elevations in the BAL from rats exposed for 12 h to 0.4 ppm O_3 each night over 5 consecutive days. No histologic data were reported. Response in BAL fluid to a subsequent single O_3 exposure had not fully recovered even after 20 days of non-exposure.

It therefore seems that 'adaptation' of some features of the inflammatory response to acute O_3 exposure occurs in rodents as well as human subjects. The mechanisms responsible for adaptation of either the lung function changes or the inflammatory changes remain to be elucidated. Wu et al. [55] recently reported that acute ozone exposure activated expression of an IL-8-like gene in monkey bronchial epithelium, but that this change regressed with continued exposure.

If such adaptive changes represent protective events, it implies that remodeling of injured airways may not occur during repeated ozone exposures unless these are appropriately spaced temporally. The borderline nature of the functional changes and relatively modest extent of morphologic alterations found in the centri-aci-

nar regions of chronically ozone-exposed rodents and primates might be related to this adaptive phenomenon. It is not known whether O_3-induced adaptation of neutrophilic inflammation also down-regulates other inflammatory responses (e.g. to infection). Such a consequence might be undesirable and also merits investigation.

Longitudinal studies of lung function [56] in residents of communities in the Los Angeles area have taken advantage of patterns of regional variation in the intensity of oxidant air pollution. They suggest an association between aging-related decline in lung function and residence in polluted areas, but have been criticized on methodologic grounds and are not broadly accepted. Another southern California study (in 6000 nonsmoking Seventh-Day Adventists) suggested that increasing cumulative exposure to ozone was associated with a trend toward increased incidence of asthma [57].

1. Conclusions

(1) Acute, reversible respiratory effects of ozone (even at levels as low as 0.08 ppm) are demonstrable in controlled exposures in healthy volunteers and in spontaneous exposures to ambient air. Both the lung function and neutrophilic inflammatory effects depend on biological amplification mechanisms. The former involves stimulation of superficial bronchial C-fibers, perhaps by prostaglandin products of arachidonate metabolism. The latter involves activation of alpha-chemokine production by oxidant-stressed epithelial cells.

(2) Patients with preexisting chronic obstructive airways disease or asthma do not appear to be exceptionally reactive to acute controlled ozone exposure. However, the interplay between ozone exposure and reaction to specific antigen in allergic individuals is under active investigation.

(3) Real world exposure to ambient air oxidant pollution is associated with increased respiratory morbidity, but the specific role of ozone in this association is unclear because of the concomitant presence of other pollutants such as particulate matter.

(4) Repeated (daily) exposures to O_3 cause progressive diminution of lung function and inflammatory responses, a phenomenon known as 'adaptation'. The inflammatory effects of O_3 exposure also appear to undergo 'adaptation'. Full restitution of inflammatory responses may then require more than 1 week, whereas restoration of lung function response occurs within 7 days. These findings should be considered in interpreting the relatively modest structural and functional changes induced by chronic exposure protocols in animal models, and suggest the need for a mechanistic understanding of adaptation and de-adaptation.

(5) In terms of protection of human health, the current NAAQS for O_3 based on 1-h average levels may be insufficient to fully take into account the fact that more prolonged exposure to O_3 levels less than 0.12 ppm is capable of causing both impaired lung function and airways inflammation. However, the ability of these reversible changes to lead to permanent airways damage remains unclear, and the need for a seasonal or annual primary standard designed to protect against irreversible lung changes has been articulated (R. McClellan, personal communication).

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Dose-effect models for ozone exposure: tool for quantitative risk estimation

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Abstract

Short-term ozone exposure causes lung function decrements, increased airway reactivity, airway inflammation, increased respiratory symptoms and hospital admissions. Exposure to long-term elevated ozone levels seems to be associated with reduced lung function (aging), increase of respiratory symptoms, exacerbation of asthma, and airway cell and tissue changes. Health risk caused by exposure to ozone has been evaluated mainly in a qualitative way by comparing ozone air quality data with health-based guidelines or standards. A preliminary approach to quantifying health risk from short-term exposure to oxidant air pollution has been taken by expert judgement, describing known or expected effects at specific levels of ozone. For quantitative assessment of the health impact of distinct ozone exposure conditions (acute, repeated daily, chronic) specific exposure-dose-response models are being developed which can be linked to human exposure data. Exposure-(dose-)response models using data from epidemiological, human-clinical and animal toxicity studies are presented.

Keywords: Ozone exposure; Exposure-dose-response models; Oxidant air pollution; Lung function

1. Human exposure to oxidant air pollution and ozone

Episodes of increased photochemical activity may last a number of consecutive days and several such episodes may occur during a summer season. The severity of oxidant air pollution is indicated by daily maximum 1- or 8-h mean ozone concentrations [1]. During oxidant air pollution episodes, elevated ozone concentrations can occur with maximal levels ranging between 100 and 400 $\mu g/m^3$ (0.05–0.2 ppm; 1 h mean). Diurnal concentration profiles of ozone shows broad peaks with mean ozone concentrations of 80–90% of the maximal 1-h average concentration during 12 h. Public health may therefore be adversely affected by both acute and repeated daily exposure to enhanced ozone concentrations for several h per day. People who live in areas frequently faced with oxidant air pollution episodes may experience adverse health effects from (sub)chronic daily exposure. Rural and urban regions with relatively high levels of photochemical activity show frequently or continuous high levels of ozone during a whole summer season.

It can be estimated that extremely large reductions in NO_x and VOC will be needed to achieve

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a considerable reduction in ozone formation. These measures are likely to be very costly and will have a large socio-economic impact. The public health consequences of not reducing ozone levels and current exposures is likely to be very serious, and possibly even more costly. Therefore, there is an urgent need for quantitative estimates of the incidence, nature, and magnitude of ozone-related acute, repeated, and (sub)chronic effects. Such a quantitative approach can also serve to found and justify possible effective control actions to reduce risks.

2. Health effects from acute ozone exposure

Health effects caused by acute exposure to ozone are frequently reviewed and discussed [2]. Controlled acute ozone exposures of humans and laboratory animals results in acute responses like lung function impairment, airway hyperresponsiveness, and airway inflammation and tissue injury [3,4]. Recently, data have been presented suggesting that airway inflammation and tissue injury also occur in people (children) exposed to photochemical air pollution [5]). Typical acute symptoms in people exposed to ozone include coughing, tightness of the chest, nausea, difficulty in breathing, and decreased ability to exercise.

A number of epidemiological studies have shown associations between acute ozone exposure and pulmonary function decrements, daily increases in morbidity, exacerbation of respiratory diseases like asthma, and increased hospital admissions and daily mortality [6–9]. Some of the effects may be indicative of the onset of long-term effects that might ultimately result in development of persistent, accelerated decline of lung function (aging), infections, asthma and chronic obstructive lung diseases, following frequently repeated or chronic exposures to ozone.

A large number of controlled studies now show effects occurring even below current ozone standards and guidelines. Because these values for short-term adverse health effects have low margins of safety, substantial doubt is growing in the scientific community whether or not these standards and guidelines still provide an adequate degree of public health protection. Moreover, ozone responses do not seem to have an apparent threshold or this threshold value is very close to background levels of ozone.

3. Critical aspects of dose-response modelling of ozone

Multiple exposures to ozone in controlled human and animal studies cause a diverging pattern of health effects, i.e. attenuation of respiratory symptoms and pulmonary function responses and progression of tissue injury, inflammatory responses and increased airway responsiveness [10–12]. Epidemiological studies have revealed some supportive data for this divergence, showing attenuation of lung function decrements in people residing in areas with frequently recurring high ambient ozone levels [13]). Controlled studies on the time course of various health effects also show that some effects like respiratory symptoms and lung function responses are maximal immediately following ozone exposure, whereas other effects, like inflammatory responses and tissue damage, may peak at a later time. Therefore, health risk analysis of acute and repeated daily ozone exposure must use exposure-response relationships selected or adjusted for specific ozone exposure situations and measuring time points.

Individuals in the population vary greatly in their biological susceptibility to acute responses to ozone and oxidant air pollution [14], showing non-responders, normal responders, and hyperresponders. It is suggested that this variation depends on both intrinsic (e.g. genetic background) and extrinsic factors (e.g. socio-economic status). It should be noted that this response variation can be different for various effects, i.e., a hyperresponder with regard to a decline in FEV_1 might be a non- or normal responder to an inflammatory reaction and vice versa. The interindividual and intraindividual differences in ozone-induced response may also be a reflection of different exposures to ozone and may therefore be related to differences in exposure variables (concentration, exposure

time, and breathing minute ventilation). Therefore, because acute ozone responses appear to depend to a major extent on the cumulative exposure and inhaled dose, people subjected to prolonged daily exposure as well as people with high breathing minute ventilation rates due to increased physical activity are suggested to be at an increased risk [15,16].

Population studies indicate that exposure to ozone during oxidant air pollution may be associated with exacerbations of asthma and increases of asthmatic visits to hospital emergency departments [17,18]. Controlled human studies indicate that, with similar changes in symptoms, lung volumes, and bronchial responsiveness in both normal and asthmatic subjects, ozone-exposed asthmatics have greater airway obstruction. In addition, asthmatics appear to have greater airway inflammation than healthy subjects [19,20]. Recent studies [21,22] also show that short-term ozone exposure increased the bronchial allergen responsiveness in subjects with mild allergic asthma. Due to the irritant nature of ozone, which is capable of inducing airway inflammation and bronchoconstriction, asthmatics and COPD patients are considered to be at increased risk for ozone exposure. Ozone may contribute to acute disease exacerbations, morbidity, and mortality.

Available human and animal toxicity data have not conclusively demonstrated gender and racial differences for pulmonary response to ozone. Data on age as a susceptibility factor are also inconclusive, although very young children are suggested to be more responsive due to greater thoracic and pulmonary doses [23], whereas older adults seem to have a decreased pulmonary function response [15].

In order to estimate the health impact of ozone, it is important to know whether the effects of ozone are interrelated and whether some of the effects are causally linked with other effects that may even be predictive in this respect. A number of studies show, however, a large dichotomy of various health effects, with lack or even inverse relationship between ozone-induced spirometric changes, respiratory symptoms, airway inflammation, and allergen responsiveness [22,24].

4. Exposure-dose-response models for short-term ozone exposure

Health risks caused by exposure to ambient oxidant air pollution have been mainly evaluated in a qualitative way by comparing air quality data with standards and health-based guidelines. However, when guidelines are exceeded, these are only qualitative indications of the likelihood of adverse health effects and do not allow any quantitative estimates of the extent and magnitude of risks.

Efforts have been made by the World Health Organization to describe air quality in terms of indicator pollutants and to compare these data with exposure-response relationships based on the judgement of experts [25]. For ambient oxidant air pollution, ozone is still considered the most important component with regard to adverse health effects in airways. Gradations have been made for all known or expected acute health effects caused by exposure at certain ozone levels, and health effects have been categorized into different classes of severity, (i.e. mild, moderate, and severe) at specified ozone concentrations.

There is an increasing need to develop and implement quantitative methods and mathematical models to estimate or predict the incidence, magnitude, and nature of health risks of ozone in the exposed general population and in specific (sensitive) subgroups. This approach has to be based, on the one hand, on exposure-dose-response models, i.e. descriptions of the relationship between exposure, dose, and experienced or predicted incidence of effects in specific human populations. On the other hand, this approach has to use exposure models, i.e. descriptions of potential and actual exposure of (sub)populations. When exposure can be related to air quality and various sources or contributive emissions, this approach can be used to calculate risk reductions dependent of improvements of air quality.

A number of experimental animal studies with acute ozone exposures has focused on establishing exposure-time-response relationships and the importance of cumulative dose, characterized by

concentration (C) and exposure time (T), for inducing airway permeability [26] and airway responsiveness [27]. The role of breathing minute volume (V_e) has only been studied to a limited extent in laboratory animal studies, showing that physical exercise, and the concomitantly inhaled dose and dose rate, may significantly enhance pulmonary deposition and related pulmonary function or permeability effects [4,28].

A number of controlled human studies with determination of various measures of lung function have also been focused on the precise inhaled (effective or delivered) dose concept of single ozone exposure. Inhaled effective dose is thereby defined as the product of C, T, and V_e. It appears that the magnitude of lung function decrements, measured directly following ozone exposure, is related to the inhaled effective (internal thoracic) dose [29,30]. It has been suggested that, both for young and old adults, the relative ranking of contributing dose variables associated with pulmonary function decline is $C > V_e > T$ [31,32].

Recently, a number of studies focused on establishing mathematical, non-linear models that describe ozone-induced decrements in lung function (FEV_1) and increases in airway permeability as a function of total inhaled dose ($C \times T \times V_e$) and dose rate ($C \times V_e$). Studies with different protocols and levels of C and T, and to a limited extent also V_e, have been evaluated for the contributive and interactive role of the various dose variables in humans [26,31,33]. Studies have also reported on the use of mathematical models describing interindividual differences in acute pulmonary function responses following ozone exposure [15,16].

5. Quantitative risk estimation

Quantitative health risk assessment of ozone exposure can provide information on the extent and magnitude of effects in the general population, specific risk groups, and individuals, if actual exposure can be estimated and exposure or dose-response relationships can be established.

With regard to the exposure model, individual exposure and inhaled dose are dependent upon a range of variables, such as geographical area, relationships between outdoor and indoor air quality, time-activity and physical activity patterns, and physiological variables. Mathematical models can be developed with functional modules that describe individual exposure parameters. These parameters can be calculated from time series of regional and urban air quality data, time-activity and physical activity patterns derived from population survey statistics, demographic data, and appropriate functions and distributions of variables, such as indoor-outdoor relationships. From these data, population frequency distributions can be estimated while preserving individual characteristics, providing the possibility to identify individuals in specific sub-populations, (e.g. the upper tail of a distribution). These types of exposure models are still in the phase of development. Ultimately, however, they can be used to estimate the exposure of the population and to predict specific health effects of interest, provided that exposure-dose-response relationships are known.

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Analysis of mutation at the *hprt* locus in human T lymphocytes

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Abstract

Studies of mutation at the hypoxanthine phosphoribosyl transferase (*hrpt*) locus in human T-cells have the potential to elucidate the molecular basis of in vivo mutagenesis, reveal exposure dependent changes in ther background frequency of mutation, and provide knowledge on individual sensitivity. Styrene exposed lamination workers in Bohemia showed a significantly higher frequency of *hprt* mutant cells than Swedish control populations studied simultaneously. In a study of 47 healthy, non-smoking male bus maintenance workers exposed to diesel exhausts, soot and oil, and 22 unexposed controls, a significant correlation ($P = 0.008$) was obtained between the levels of aromatic DNA adducts and frequencies of *hprt*-mutant T-cells. In the group of workers with the highest exposure, subjects with glutathione *S*-transferase (GSTM1) deficiency showed significantly higher ($P < 0.05$) frequency of *hprt* mutant T-cells than GSTM1-positive subjects. The highest adduct levels were found in subjects with the combined genotype of GSTM1 and NAT2 deficiency (GSTM1-negative slow acetylators). These results indicate that GSTM1 and NAT2 genotypes may play a role in determining the individual levels of *hprt* mutation and DNA adducts. Using PCR-based screening methods, *hprt* mutations have been classified in 462 T-cell clones from 43 subjects in this study population. Deletions were found in 3% of the mutants, coding errors in 81% and splice mutations in 17%. Transitions and transversions were equally common, and all types of base substitutions were detected.

Keywords: Gene mutation; *hprt* locus; Human T lymphocytes; DNA adducts; Genetic predispositon

1. Introduction

Several methods are available for the analysis of human somatic mutation in vivo (review in [1,2]). The most versatile of these methods measures the frequency of mutation at the X-linked hypoxanthine guanine phosphoribosyl (*hprt*) locus in T lymphocytes [3,4]. This system has several unique advantages.

Firstly, in vivo mutations at the *hprt* locus can be analysed both in somatic cells (i.e. T lymphocytes) [5] and in the germ line (i.e. in patients with the Lesch-Nyhan syndrome) [6–8], offering the possibility to study mutational mechanisms in germ cells by inference from data derived from somatic cells.

Secondly, spontaneous and chemically or radiation-induced *hprt* mutations can be studied

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in T cells in vitro [9–13], which allows experimental approaches to be used in close similarity to the in vivo situation.

Thirdly, since the entire nucleotide sequence of the *hprt* gene is known [14], it is possible to identify almost all different *hprt* mutations that give rise to *hprt* deficient phenotype.

Finally, the specificity of T-cell receptor (TCR) gene rearrangement makes it possible to establish the identity of each individual mutant T-cell clone, so that identical mutations in cells with dissimilar TCR gene rearrangements can be ascribed to different mutational events [15].

Taken together, these features make the analysis of *hprt* mutation in T lymphocytes an important source of knowledge regarding molecular mechanisms of mutagenesis in human cells (review in [16]). Moreover, it is a promising system for monitoring pollutant-induced genetic damage in the human population, and an interesting surrogate system for studies of early mutational events in carcinogenesis.

2. *hprt* mutant frequencies in human populations

Extensive information on human *hprt* mutation has already been collected in an international database [17] and presented in several comprehensive reviews [2,18,19]. Measurements of mutant frequency (MF, i.e. the frequency of *hprt* mutant peripheral blood T lymphocytes) in groups of healthy individuals in different laboratories show a remarkable similarity in group mean MF among adults, in spite of a 10–20-fold interindividual variation [2]. As shown in Table 1, this is also evident in some recent study populations from the United States, as well as in

Table 1
hprt mutant frequencies in T cells in different populations

Study populations	No. of subjects	MF × 10^6 mean ± S.D. (range)	CE (% ± S.D.)	Age mean (range)	Reference
Sussex, UK					
Normal adult non-smokers	49	6.6 (1–32.2)	58	38 (18–84)	[2]
North Carolina, USA					
Healthy volunteers					
Male non-smokers	34	7.1 ± 5.0 (1.4–26.1)	29 ± 12	28 (19–41)	
Female non-smokers	28	8.1 ± 7.5 (1.9–36.5)	33 ± 16	31 (22–45)	[22]
Vermont, USA					
Laboratory and office personnel	21	7.4 ± 5.5 (1.8–24.8)	36 ± 16	28 (19–43)	[54]
Sweden					
All non-smokers					
Bus maintenance workers	47	8.6 ± 4.7 (1.9–22.6)	59 ± 27	43 (27–65)	
Fine mechanics and laboratory personnel	22	8.4 ∓ 84.9 1.4–22.5)	58 ± 25	38 (23–61)	[34]
Bohemia, Czech Rep.					
All non-smokers:					
Lamination workers (styrene exposed)	9	17.5 ± 12.3 (8.4–49.0)	43 ± 11	44 (32–51)	
Factory workers	7	15.7 ± 8.3 (6.8–31.6)	42 ± 12	42 (32–52)	
Laboratory personnel	8	11.8 ± 6.8 (7.3–27.4)	39 ± 8	44 (30–51)	[28]

our own data on healthy, non-smoking, bus-maintenance workers, fine mechanics and laboratory controls from Sweden. Slightly higher group mean *hprt* MFs were obtained in our study of styrene-exposed lamination workers and the corresponding laboratory controls from Bohemia. These data will be described in some more detail below.

Repeat sample analyses suggest that the methodological error of *hprt* MF measurement in T cells using the clonal assay is relatively small [20], although the assay in different laboratories may differ with regard to cell priming, counting and plating procedures, feedercells and growth supporting media. However, if the cloning efficiency (CE), which in most studies is inversely related to the MF, is kept at sufficiently high level (0.3–1), the estimate of MF appears to be reliable [20].

The large interindividual variation of MF in healthy subjects is partly related to age and smoking habits. In most studies, the MF has been shown to increase by 1–3% per year in adults (review in [2]), and some laboratories have reported the MF to be 20–50% higher in smokers than in non-smokers (e.g. [21,22]). Recent data with regard to the effect of dietary habits [23] need further confirmation.

Medical treatments such as radiation and chemotherapy, as well as conditions associated with defective DNA repair capacity (xeroderma pigmentosum) or genomic instability (ataxia telangiectasia and Fanconi's anemia) have been shown to be associated with an increased MF in several studies (review in [2]). However, few studies have been able to show a convincing effect of environmental and occupational exposures on *hprt* MF in peripheral T cells (e.g. [24–28]). Unknown background factors may contribute to the large interindividual variations in MF, and relatively small increases in the average MF due to environmental exposures may be difficult to observe unless the exposure is well defined with regard to internal dose and duration.

There are several host factors which theoretically could influence the background MF in T cells of healthy individuals, such as genetically determined polymorphisms of enzymes involved in DNA repair functions or the activation and detoxification of endogeneous and environmental mutagens and carcinogens. The hypothesis behind this concept is that individuals with enhanced metabolic activation and/or decreased detoxification or DNA repair capacity, will acquire higher levels of DNA damage and therefore show increased mutation rate. To investigate this possibility, we have studied the relationship between *hprt* MF, possible susceptibility genotypes and levels of DNA adducts in peripheral T cells from groups of healthy individuals with various types of occupation.

3. *hprt* MF and DNA adducts in styrene-exposed lamination workers

Styrene is a possible human carcinogen according to the IARC classification [29], and one of the most important chemicals in the manufacture of plastic material. Protein and DNA adducts, as well as DNA strand breaks and cytogenetic changes have been reported previously in blood samples from styrene-exposed workers (reviews in [30,31].

hprt MF and styrene-specific O^6-guanine DNA adducts were studied in a group of styrene-exposed Bohemian lamination workers [28]. Four blood samples were taken over a 7-month period. The first and second samplings were separated by a 2-week vacation time. Exposure was monitored both with regard to ambient air concentrations and internal dose biomarkers. Styrene-specific O^6-guanine DNA adducts were measured with a modified ^{32}P-postlabeling technique [32,33]. Factory workers, employed in the same factory as the lamination workers, but not occupied with lamination work, were used as controls. During the course of the investigation, it became apparent that the factory workers also showed increased levels of O^6-guanine adducts, and another control group of laboratory personnel, without any known styrene exposure, was recruited. Blood samples from the laboratory controls were taken at one time point only (sampling IV).

The average *hprt* MF was found to be 1.5–2

times higher in the Bohemian study population than in the Swedish study populations (Table 1), although these studies were carried out in the same laboratory and during the same time period, using the same experimental procedures [34,35]. This relatively large difference in group mean MF, in spite of similar age distribution, smoking habits (all are non-smokers), and CE values (Table 1), indicate that *hprt* MF is affected by other, so far unknown background factors, perhaps related to cultural and social conditions and dietary and other lifestyle factors. Such possible regional and perhaps ethnic differences in somatic mutation rate need to be studied in more detail.

The *hprt* MF in the lamination workers were higher than in the factory controls at 3 of the 4 sampling times, but the differences were not statistically significant (Table 2). However, there was a small, but significantly higher *hprt* MF in the laminators compared to the laboratory controls at sampling time IV, the only time point when these data could be compared, since samples from both groups were collected within the same week and analysed simultaneously (Table 2). This result indicates that styrene exposure is associated with an increase in *hprt* mutation in human T lymphocytes.

The DNA adduct levels were remarkably constant over the 4 sampling times, and significantly higher in the laminators than in the factory controls. In the laboratory controls, the levels of styrene-specific DNA adducts were on the border of detection (Table 2). Thus, on a group mean basis, the DNA adduct levels showed the same relationship between study groups as did the *hprt* MF, being highest in the laminators and lowest in the laboratory controls. However, there was no correlation between the individual levels of DNA adduct and *hprt* MF, suggesting that there is no simple, quantitative relationship between these two biomarkers. The possible reason for this lack of correlation may be that the measured DNA adduct levels, which appear to be very stable since they do not change before (sample I) and after vacation (samples II–IV), reflect a non-repaired fraction of the adducts remaining in non-active parts of the genome. Since measurements of DNA adducts are based on the whole genome, while *hprt* mutations are specific locus changes in an active gene occupying only 55 kb of the genomic DNA, the rate and extent of removal of adducts and their distribution in the genome may very well affect the relationship between the two markers.

Previous studies have shown that many chemicals and radiations induce specific patterns of mutation at the *hprt* locus in T cells, which are readily distinguished from the background or spontaneous pattern of *hprt* mutation (e.g.

Table 2
hprt MF and styrene-specific O^6-guanine DNA adducts in laminators and controls

Group and No. of subjects	Sample I	Sample II	Sample III	Sample IV	All samples
Laminators (9)					
MF $\times 10^6$	20.7 ± 25.9	18.8 ± 15.8	15.3 ± 5.9	18.0 ± 5.2**	17.5 ± 12.3
Adducts/10^8 dNp*	5.0 ± 2.9	5.9 ± 2.0	6.0 ± 2.0	4.8 ± 2.5	5.4 ± 1.7***
Factory workers (7)					
MF $\times 10^6$	17.2 ± 5.5	14.7 ± 11.2	18.1 ± 13.3	12.8 ± 7.2	15.7 ± 8.3
Adducts/10^8 dNp*	1.4 ± 0.9	0.7 ± 0.4	0.9 ± 0.6	0.8 ± 0.4	1.0 ± 0.4***
Laboratory personnel (8)					
MF $\times 10^6$				11.8 ± 6.8**	11.8 ± 6.8
Adducts/10^8 dNp*				0.3 ± 0.5	0.3 ± 0.5

Data from [28].
* Data show group mean ± S.D.
** $P < 0.02$ for difference between laboratory personnel and laminators.
*** $P < 0.001$ for difference between laminators and factory workers.

[12,36,37]). However, the relatively small difference in MF between styrene-exposed lamination workers and unexposed controls would make it very difficult to detect any difference in the mutation pattern in the 2 groups. In order to study the possible specificity of styrene-induced mutation, we therefore took advantage of the possibility offered by the *hprt* assay, to study mutation induction in T cells in vitro, i.e. in the same human cell population as was studied in vivo.

A series of in vitro experiments was carried out [13] in which T lymphocytes from healthy blood donors were exposed to styrene-7,8-oxide (SO), the major in vivo metabolite of styrene which is thought to be responsible for its mutagenic and probably carcinogenic in vivo effect [29]. Both *hprt* MF and styrene-specific O^6-guanine adducts were measured. SO treatment at a concentration of 0.2–0.4 mM for 24 h resulted in a dose-dependent decrease of cell survival, and increase of *hprt* mutants and O^6-guanine adducts. Higher SO concentrations caused pronounced cell death and a further increase of DNA adduct levels, but a decrease of *hprt* MF (Table 3).

Thus, again there was no simple relationship between *hprt* MF and O^6-guanine adduct levels. Moreover, these in vitro results suggest that exposure to comparatively high SO doses for short duration in vitro is relatively inefficient in inducing *hprt* mutation and O^6-guanine adducts in comparison to chronic, low-dose exposure to styrene in vivo. A possible explanation for this is that both O^6-guanine adducts in lymphocyte DNA and *hprt* mutant T cells accumulate with different kinetics over considerable time periods in vivo.

4. *hprt* MF and aromatic DNA adducts in bus maintenance workers

Occupational exposure to diesel exhausts, which contain a number of mutagenic polyaromatic hydrocarbons (PAHs) and nitro-PAHs, has been associated with an increased risk of lung cancer (review in [38]), and increased levels of aromatic DNA adducts in peripheral lymphocytes [39]. In a recent work, Perera et al. [27] showed a significant correlation between PAH-DNA adduct levels and *hprt* MF in a group of foundry workers. Thus, further studies are warranted to establish the relationship between exposure-related DNA damage and effect-related genetic change in human cells in vivo.

Aromatic DNA adduct levels and *hprt* MF in T cells were studied in 47 bus maintenance workers and 22 control subjects [34]. All were non-smokers. The bus maintenance workers were exposed to diesel exhaust, soot and lubricating oils. Within this group, 16 subjects were garage workers considered to be highly exposed to diesel exhausts, 25 subjects were mechanics mainly exposed to engine and lubricating oils, and 6 were low-exposed workers with 'other' maintenance work. The controls were 11 fine mechanics from a hospital workshop and 11 laboratory personnel. DNA adduct levels in lymphocyte DNA were measured by ^{32}P-post-

Table 3
hprt MF and levels of O^6-guanine DNA adducts in human T cells treated with SO for 24 h in vitro

Concentration of SO (mM)	Survival (%)	MF × 10^6 (mean ± S.D.)[a]	Adducts/10^8 dNt mean (range)
0	100	4.3 ± 0.8	0
0.2	78	15.0 ± 7.2	1.5 (0.98–2.04)[b]
0.4	43	15.7 ± 9.4	2.5[c]
0.6	8.4	6.2 ± 1.6	3.1 (2.59–4.05)[b]

Data from [13].
[a] Five control and 3 SO cultures at each dose level.
[b] Two experiments.
[c] One experiment.

labelling [39], and *hprt* MF by the T-cell cloning assay [35].

In both study groups, the CE was high (mean 59%), showed no decrease with age and was negatively correlated with MF. A highly significant correlation between MF and age was obtained, indicating an increase of MF by 2.5% per year. However, no significant difference in MF was observed between the workers and controls. In fact, both mean and range of MF were very similar to data reported for other 'healthy, non-exposed control' groups (c.f. Table 1).

The *hprt* MF was highest in the most heavily exposed group of garage workers, and lowest in the small group of workers occupied with 'other' work tasks. However, these differences were not statistically significant. The aromatic DNA adduct levels ranged from 1.6–6.5/10^8 dNt. There was no correlation between adduct levels and age, but a statistically significant difference in adduct levels was obtained between workers and controls, and between the garage workers and the other 2 groups of workers (Table 4).

A significant correlation was obtained between the aromatic DNA adduct levels and *hprt* MF for the exposed workers ($P = 0.04$) as well as for the entire study population ($P = 0.008$), but not for the smaller group of controls. However, the trend for a positive correlation between *hprt* MF and DNA adduct levels was very similar in the control group and in the group of workers (Fig. 1), suggesting that aromatic DNA adduct levels are indeed related to the mutation frequency.

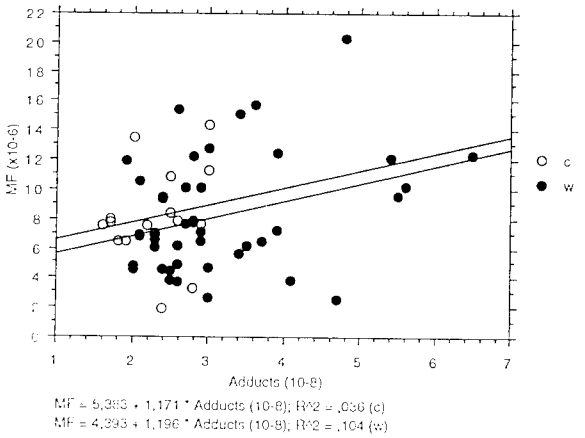


Fig. 1. Correlation between *hprt* MF and aromatic DNA adduct levels in bus maintenance workers and controls (data from Hou et al., 1995a). ●, workers (w); ○, controls (c). The MFs have been adjusted for age. The solid lines represent the best fit regression equations below the figure.

Since there was no difference in the mean *hprt* MF between the groups, it is possible that the controls, consisting of fine mechanics and laboratory personnel, are exposed to other mutagens, not detected by the assay which was used for DNA adduct measurements.

5. The relationship between *hprt* MF and genotypes for glutathione *S*-transferase μ and *N*-acetyltransferase 2

Several studies indicate that polymorphisms of enzymes involved in carcinogen metabolism, e.g. cytochrome P450 1A1, glutathione *S*-transferase

Table 4
hprt MF and aromatic DNA adduct levels in bus maintenance workers and controls

Study groups	No. of subjects	MF × 10^6 (mean ± S.D.)	Adducts/10^8 dNt (mean ± S.D.)
Bus maintenance workers			
All subjects	47	8.6 ± 4.7	3.2 ± 1.0*
Garage workers	16	9.7 ± 5.8	3.6 ± 1.3
Mechanics	25	8.5 ± 4.1	3.0 ± 0.9
Others	6	6.4 ± 2.8	2.6 ± 0.5
Controls			
All subjects	22	8.4 ± 4.9	2.3 ± 0.5*
Fine mechanics	11	9.6 ± 5.3	2.3 ± 0.5
Laboratory personnel	11	7.2 ± 4.5	2.3 ± 0.5

Data from [34].
* $P < 0.001$ for difference between bus garage workers and controls.

μ (GSTμ) and *N*-acetyltransferase 2 (NAT2), may confer susceptibility to cancer development in response to environmental and occupational exposure in the normal population (review in [40]).

GSTμ is engaged in the detoxification of many chemical mutagens, including PAHs. About 50% of the Scandinavian population [41] are homozygous for a total deletion of the GSTM1-gene, causing complete GSTμ deficiency. Increased frequencies of GSTM1(−/−) subjects have been recorded among patients with cancer of the lung, stomach, colon and bladder [42–46].

NAT2 is generally considered to be a detoxifying enxyme, since by *N*-acetylation of aromatic amine procarcinogens it competes with *N*-oxidation, which may produce DNA-reactive intermediates. The acetylation polymorphism is due to the occurrence of at least 8 variant NAT2 alleles, 2 'rapid' and 6 'slow'. The slow acetylation phenotype occurs in subjects with 2 slow alleles (s/s), and rapid acetylation in subjects with 2 rapid (r/r) or 1 rapid and 1 slow (r/s) allele. Slow acetylation has been associated with bladder cancer, and rapid acetylation with colorectal cancer (reviews in [47,48]). In a recent study, slow acetylators were found to have higher levels of 4-aminobiphenyl-hemoglobin adducts than rapid acetylators [49]. The genotype frequency for slow acetylators (s/s) is about 60% in the Scandinavian population (Hou et al., unpublished data).

It was hypothesized that subjects with GSTM1(−/−) and NAT2(s/s) genotypes, due to their less effective detoxification ability, may acquire higher levels of DNA adducts and *hprt* mutations, albeit such effects may develop over time, and depend on the level of exposure and, for example, smoking habits. To test this hypothesis we determined the GSTM1 and NAT2 polymorphisms in the healthy, non-smoking bus maintenance workers and the corresponding control group presented above, using polymerase chain reaction (PCR)-based screening techniques [34].

In the study population as a whole, GSTM1(−/−) subjects showed a 20% higher mean *hprt* MF than the GSTM1-positive individuals, but this difference was not statistically significant. In the group of garage workers with the highest exposure, 8 subjects with GSTM1(−/−) genotype were found to have a significant increase ($P < 0.05$) of *hprt* MF as compared to the 8 GSTM1-positive individuals (Fig. 2). No difference with regard to NAT2-genotype was observed. However, when the combined genotypes were considered, the 18 subjects with GSTM1(−/−) and NAT2(s/s) genotype showed a higher increase of MF by age (3.1%/year) as compared to the other 3 genotype combinations (2.4–2.5%/year), although the difference was not statistically significant. The GSTM1-negative slow acetylators also showed the highest adduct levels [34].

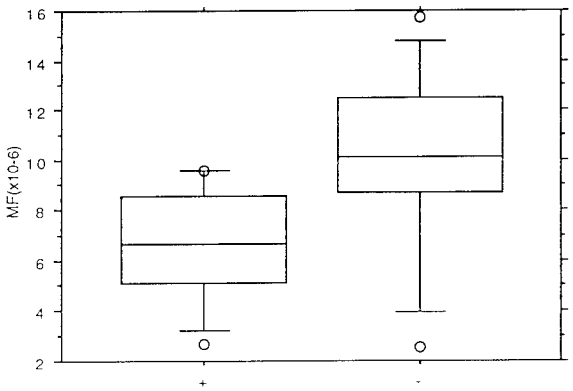


Fig. 2. Box plot of *hprt* mutant frequencies in T lymphocytes of GST M1-negative and GSTM1-positive bus garage workers (data from Hou et al. [34]). +, the 8 GST-positive subjects; −, the 8 GST-negative subjects; box, 25th, 50th (median) and 75th percentiles; bars, 90th and 10th percentiles; ○, outliers. The MFs have been adjusted for age. The *P* value for the difference between the groups is $P < 0.049$.

Taken together, these results indicate that GSTM1 and NAT2 genotypes may play a role in determining the individual levels of *hprt* MF and DNA adduct levels. However, it is obvious that larger study populations with well-defined exposure history will be needed to establish these relationships in quantitative terms.

6. Types of mutation in bus maintenance workers and controls

The significant correlation between aromatic

DNA adduct levels and *hprt* MF in the bus maintenance workers suggested a possible role of these adducts in mutation induction. Thus, it was of interest to study the patterns of *hprt* mutation in these subjects. A total of 462 mutant T-cell clones, approximately 10 clones from each of 29 exposed workers and 14 control subjects were collected, and analysed by multiplex PCR for genomic alterations, and by reverse transcriptase PCR for splicing and coding errors [50].

There was no difference between the exposed and control groups with regard to the frequency of deletions, splicing mutations and coding errors. In total, 2.6% of the mutants showed a genomic deletion, 23% were splicing mutations and 75% were coding errors. It was not possible to classify 135 of the 462 mutants (29%) by this crude screening method, since the multiplex PCR yielded a normal band pattern, whereas no RT-PCR product was obtained (Table 5). It is possible that a substantial part of these clones contain splicing mutations which gives rise to unstable or very low levels of *hprt* mRNA.

By completion of the molecular analysis of this set of mutants from a relatively homogeneous and well-characterized human population, it will be possible to compare the patterns of *hprt* mutation in different study populations (e.g. [51–54], and gain further insight into background factors which determine the relationship between somatic mutation, genetic polymorphisms and DNA adduct levels.

7. Conclusions

Analysis of *hprt* mutant cells in peripheral blood by the T-cell cloning assay is a promising system for the study of human somatic gene mutation. However, the great interindividual variations in background frequency of *hprt* mutation need to be explained. As shown in the present studies, polymorphisms of metabolizing enzymes such as GST M1 and NAT2 may have some influence on the MF. There are many other candidate enzymes involved in metabolism and DNA repair which remain to be studied in this respect.

The relationship between DNA adduct levels and patterns and frequencies of mutation is an important concept, since it may link environmental exposure with genetic effect at the molecular level. However, this relationship is not likely to be simple, since both adducts and mutations are affected by a number of independent variables, such as chemical stability and DNA repair in the case of adducts, and the complexity and turnover of the peripheral lymphocyte population in the case of the mutations. Thus, no relationship

Table 5
Types of *hprt* mutation in bus maintenance workers and controls

Method of analysis[a]	No. of mutants (%)	Types of mutation
MP-PCR		
Total studied	462 (100%)	
Abnormal	12 (2.6%)	Deletion/rearrangement
RT-PCR		
Total studied	462 (100%)	
RT-PCR negative	139 (30%)	
Normal MP-PCR	135	Not classified
Abnormal MP-PCR	4	Deletions (same as above)
RT-PCR positive	323 (100%)	
Altered *hprt* cDNA		
Normal MP-PCR	74 (23%)	Splicing mutations
Abnormal MP-PCR	8	Deletions (same as above)
Normal *hprt* cDNA	241 (75%)	Coding errors

Data from [50].
[a] MP-PCR, multiplex PCR; RT-PCR, reverse transcriptase PCR.

between O^6-guanine adducts and *hprt* mutation was observed, neither in the styrene-exposed laminators, nor in the styrene oxide-exposed cells in vitro, although both biomarkers did increase in response to exposure.

In contrast, there was an significant correlation between aromatic DNA adducts and *hprt* mutation in the bus maintenance workers, and similar results have been reported for foundry workers [27]. However, none of these studies showed a significant effect of the exposure on the *hprt* MF.

It is clear that further studies are needed to explain these observations, and to improve our knowledge about background factors, environmental as well as host factors, which influence the frequency and rate of somatic mutation in vivo.

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Toxicology Letters

A microassay for measuring cytosine DNA methyltransferase activity during tumor progression

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Abstract

The cytosine DNA methyltransferase (MT) enzyme, which catalyzes DNA methylation at CpG sites, is overexpressed at the mRNA level during the progressive stages of colon cancer. This paper describes the adaption of a sensitive microassay for determining MT enzyme activity during tumor progression in human colon and murine lung. MT activity was progressively elevated in mucosa from familial adenomatosis polyposis patients, mucosa adjacent to cancers, and in colonic adenocarcinomas when compared to colonic mucosa from control patients. In addition, the activity of this enzyme was increased in alveolar type II but not Clara cells isolated from A/J mice following carcinogen exposure and continued to increase during tumor progression. The use of a microassay for measuring MT activity indicates that changes in enzyme activity were in general agreement with previous findings of increased MT mRNA levels during colon cancer progression and also implicates the involvement of this pathway in lung cancer development.

Keywords: DNA methyltransferase activity; Colon cancer; Lung cancer; Tumor progression; Type II cells

1. Introduction

5-Methylcytosine modification of mammalian DNA, both in CpG islands located in promoter regions and in exons, is important in gene regulation. A decrease of 5-methylcytosine in 5′ flanking regions or in exons of genes has been associated with increased gene transcription, while an increase in 5-methylcytosine can result in the silencing of gene expression [1]. DeBustros et al. [2] have observed substantial hypermethylation within specific regions of chromosome 11p in small cell lung cancers and lymphomas. This hotspot for methylation is thought to contain putative tumor suppressor genes. Furthermore, in one chromosome 11 gene examined, calcitonin, the increased methylation in the tumor cells coincided with the presence of an 'inactive' chromatin pattern in the transcriptional regulatory area [3]. Distinct hypermethylation at 17p and 3p has also been observed in lung and colon cancers [4]. The hypermethylated locus on 17p was mapped to 17p13.3. The region containing the hypermethylated locus has also been recently cloned and sequenced [5]. The gene identified, HIC-1 (hypermethylated in cancer), is a strong candidate for a tumor suppressor gene at this locus.

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Abnormal methylation has also been examined during the progressive stages of colon cancer. Makos et al. [4] found that the methylation of the 17p13.3 locus harboring the HIC-1 gene was detected in premalignant colonic adenomas and increased in extent during colon cancer progression. Moreover, hypermethylation at this locus was found to precede allelic loss. Thus, these studies suggest that methylation patterns may change within specific genes. Furthermore, subsequent gene inactivation within these chromosomal loci may occur prior to clinical cancer and contribute to the development, clonal expansion, and progression of initiated cells.

The cytosine DNA methyltransferase (MT) enzyme catalyzes DNA methylation at CpG sites. It establishes normal methylation patterns during embryogenesis and reproduces these patterns during replication of adult cells [6]. MT expression is 18-fold higher in colon cancers than paired adjacent mucosa and 13-fold higher in normal-appearing mucosa from cancer patients than in mucosa from individuals without tumors [7]. Thus, increases in MT activity may mediate abnormal regional increases in DNA methylation that could lead to gene inactivation and chromosome loss. DNA methylation could also result in changes in DNA sequences, because methylated cytosine is a highly mutable base in the eukaryotic genome [8].

The studies described above have used changes in gene expression detected largely by Northern analysis and the reverse transcribed polymerase chain reaction (RT-PCR) to measure MT expression. The DNA MT gene is known to be controlled by both posttranscriptional [9] and posttranslational modifications [10]. The ability to quantitate the activity of this enzyme following carcinogen exposure and in microdissected lesions during tumor progression would be invaluable for characterizing the involvement of this process in human cancers. This paper describes the adaption of a sensitive microsassay for examining DNA MT activity during tumor progression in 2 different models, human colon and murine lung.

2. Materials and methods

2.1. DNA MT assay

A modification of the assay developed by Adams et al. [11] was used to determine DNA MT activity [12]. All tissues and cells were frozen in liquid nitrogen immediately following resection or isolation and then stored at −70°C until use. Three-tenths to 5 mg tissue or 1×10^6 cells were homogenized using a glass pestle in a 2.2-ml microfuge tube containing 500 μl lysis buffer (50 mM Tris (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, 0.01% sodium azide, 6 mg/100 ml phenylmethyl sulfonyl fluoride, 10% glycerol, and 1% Tween 80). This suspension was passed through a 25-gauge needle, frozen to −70°C, and then thawed to 37°C. The freeze-thaw cycle was repeated 3 times. Protein concentration was determined by the Bradford assay.

Cell lysates containing 5 μg protein were mixed with 0.5 μg poly deoxyinosine-deoxycytidine (dI-dC) template (Pharmacia, Inc., Piscataway, NJ) and 3 μCi ^{3}H *S*-adenosyl methionine (SAM, Amersham, Arlington Heights, IL) in a total volume of 20 μl and incubated at 37°C for 2 h. Reactions were stopped, protein extracted, and the poly dI-dC template recovered by ethanol precipitation as described [12]. RNA was removed by resuspension of the precipitates in NaOH; DNA (poly dI-dC) was spotted on Whatman filters, dried, then washed with trichloroacetic acid followed by 70% ethanol. Filters were placed in scintillation cocktail and MT activity, measured as ^{3}H incorporation, was determined by scintillation counting. Because of differences in the radiochemical purity of the SAM, variations in MT activity were observed across assays. Therefore, each experiment was standardized using a positive control (whole cell lysate from HT1080, a cell line derived from a human osteosarcoma). In order to exclude background protein activity and RNA methylation, all samples were assayed with a negative control containing the whole cell lysate, but no poly dI-dC template. Results were expressed as dpm/μg protein. All assays were performed in duplicate. Limit of detection was 20 dpm above background

levels. Statistical assays were performed using a Student's *t*-test.

2.2. Isolation of lung cells and microdissection of tumors

A/J mice, 6–8 weeks old (Jackson Laboratory, Jackson, MS) were treated 3 times (every other day) with the tobacco-specific carcinogen 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK, 50 mg/kg, i.p.). Animals were sacrificed 1, 3, 7, and 14 days after treatment. Lungs from 18 mice were pooled for isolation of alveolar type II and Clara cells as described [13]. A lung cell digest was obtained by instilling a 1% solution of protease I (Sigma Chemical Co., St. Louis, MO) via the trachea into lungs that were cleared of blood by perfusion with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered salt solution (HpBS). Inflated lungs were incubated with protease for 5 min at 37°C and the digested tissue minced into small pieces (approximately 1 mm^3) with scissors. The volume of the cell suspension was increased to approximately 75 ml by adding HpBS:F12K medium (2:1) containing bovine serum albumin (1%), soybean trypsin inhibitor (100 mg, Sigma), and DNAse I (50 mg, Sigma). This cell suspension was degassed, stirred to dissociate clumps of cells, and filtered consecutively through 2 layers of cheesecloth, 1 layer of 160-μm nylon mesh, and 1 layer of 40-μm nylon mesh. This cell suspension was layered onto fetal bovine serum (Sigma) and centrifuged for 12 min at $200 \times g$. The cell pellet was resuspended in HpBS:Hams F12K medium containing 0.05% DNAase I. Enriched populations of alveolar type II and Clara cells were then isolated from the cell digest by centrifugal elutriation using a Standard chamber (Beckman, Fullerton, CA) and further purified by a second elutriation using a Sanderson chamber. Alveolar type II and Clara cells were 71 ± 2 and 72 ± 2% pure, respectively, by this procedure. Three aliquots of type II and Clara cells (1×10^6 each) were frozen at -80°C in lysis buffer. The remaining cells were pelleted and frozen at -80°C for future studies.

Lung tumors were induced [14] by the treatment of mice 3 times a week for 7 weeks with NNK (50 mg/kg, i.p.). Tumors were microdissected from lungs 20, 28, 36, 44, and 52 weeks after initiation of treatment, frozen in liquid nitrogen, and stored at -80°C.

3. Results and discussion

3.1. Standardization of the DNA MT assay

The MT assay was standardized with respect to incubation time and cellular protein. The enzymatic activity was linear through 4 h of incubation at 37°C ([12], data not shown) and between 1 and 8 μg protein (Fig. 1). Linearity was observed with protein obtained from HT1080, alveolar type II, and Clara cells, and lung tumors. The plateau in activity was not due to depletion of the poly dI-dC template or SAM, because increasing amounts of these substrates did not affect the rate of product formation [12]. This plateau most likely results from the natural breakdown product of SAM, *S*-adenosyl-homocysteine, which is a strong inhibitor of all transmethylation reactions [15]. To avoid any plateau effect, assays were conducted with 5 μg protein for 2 h.

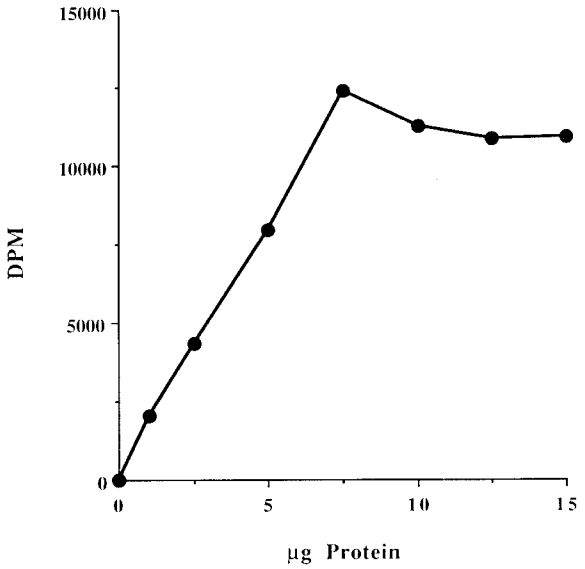


Fig. 1. Standardization of the DNA methyltransferase assay. Cellular protein (1–12 μg) from HT1080 cells was incubated with substrates as described. All samples were assayed in duplicate. Typical experiment.

3.2. DNA MT activity during colon cancer progression

DNA MT activity was determined in 15 matched-pair specimens of colonic adenocarcinomas and adjacent, uninvolved mucosa. MT activity was also determined in normal-appearing colonic mucosa of 11 control patients and in mucosa from 11 patients with familial adenomatous polyposis (FAP). MT activity, when compared to mucosa of control patients, was significantly ($P < 0.05$) elevated 1.4-, 1.6-, and 5.4-fold in the mucosa from FAP patients, mucosa adjacent to cancers, and in colonic adenocarcinomas, respectively (Fig. 2). MT activity was also measured in an established colon carcinoma, a benign adenomatous polyp, and uninvolved mucosa from one patient. Enzyme activity increased in parallel to progression of the disease in this patient. The greater difference for DNA MT transcript levels compared to MT activity during the progressive stages of colon cancer could stem from the assays employed. The RT-PCR assay that was used to determine transcript levels is only semiquantitative and may over-amplify the differences in transcript levels between normal mucosa and adenocarcinomas. Alternatively, posttranscriptional and posttranslational modifications of the MT gene could account for the observed differences. However, the changes in MT activity were in general agreement with previous findings of increased MT mRNA levels during colon cancer progression [7] and, therefore, support the use of this microassay as a tool for characterizing MT activity during initiation and tumor progression.

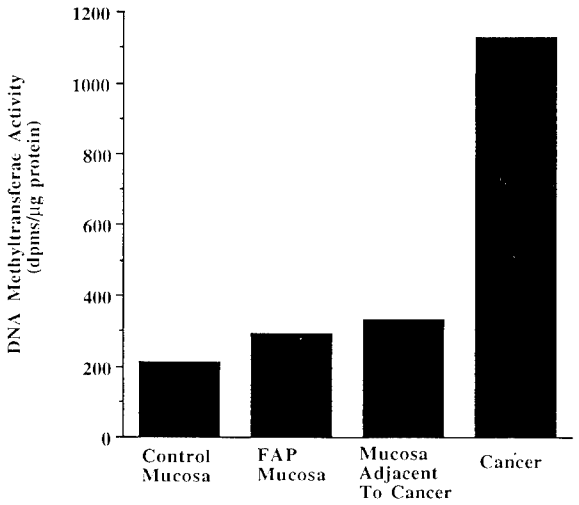


Fig. 2. DNA methyltransferase activity in colon cancer and mucosa. MT activity was determined as described in Materials and methods. Values are means from 11–15 samples. The range of MT activity for each group is described in Ref. [12].

3.3. DNA MT activity following carcinogen exposure and during lung tumor progression

The A/J mouse is a proven model for studying tumor progression. Treatment of these mice with NNK induced lesions that were first detected as alveolar epithelial hyperplasias involving alveolar type II cells. The majority of these hyperplastic lesions progress to pulmonary adenomas, then to carcinomas [16]. In addition, one genetic alteration that is frequently detected in the hyperplasias and tumors is activation of the K-*ras* gene by a GGT to GAT mutation in codon 12 [16,17]. Activation of the K-*ras* gene is also involved in 30% of human pulmonary adenocarcinomas [14]. Although benign tumors are rarely detected in human lung cancer, studies by Auerbach et al. [18] demonstrated a high frequency for premalignant histologic changes that ranged from hyperplasia and metaplasia to severe dysplasia throughout the bronchial mucosa from cigarette smokers. Thus, this murine model was used to characterize MT activity in the lung following carcinogen exposure and during tumor progression.

MT activity was determined in target (type II) and nontarget (Clara cell) cells for lung tumor development in A/J mice following treatment with NNK. Endogenous activity of MT was similar between type II and Clara cells. One day following treatment, MT activity was increased significantly in type II cells and continued to increase for at least 1 week. No effect on MT activity following NNK treatment was observed in Clara cells. Moreover, enzyme activity was 4 times greater in type II cells than in Clara cells 1 week after cessation of NNK (Belinsky, unpublished).

The mass distribution of lesions analyzed for MT activity varied from 0.3–5.0 mg. Based on

the previously established tumor progression model [16], lesions with the smaller mass (0.3–0.5 mg) were predominantly hyperplasias and small adenomas; adenomas and carcinomas were most prevalent in the 0.6–1.5 and 3.1–5.0 mg size ranges, respectively. The enzyme activity increased in direct proportion to the size of the lesions (Belinsky, unpublished). This increase in MT activity during murine lung tumor progression paralleled that observed in human colon cancer [7]. The marked increase in MT activity in type II cells and during tumor progression may modulate the methylation state of key regulatory genes and promote the selective clonal expansion of *ras* initiated cells.

4. Conclusions

The use of a microassay for measuring MT activity has advantages over Northern analysis and RT-PCR. It allows the quantitation of enzyme activity, thereby eliminating posttranscriptional and posttranslational influences on processing of DNA and RNA. The assay can also be conducted with small amounts of tissue (0.3 mg) or cells (1×10^6) which facilitate the study of polyps, dysplastic and metaplastic lesions, and carcinoma in situ.

The use of a microassay for measuring MT activity has revealed that changes in the activity of this pathway occur prior to the development of alveolar hyperplasia and are localized to the type II cells of the A/J mouse lung. The detection of increased MT activity in this murine lung cancer model further supports the hypothesis that deregulation of this process may serve to promote genomic instability and contribute to the progressive chromosomal losses and gene-specfic methylation seen in human cancer. Recent studies have implicated changes in methylation in affecting the function of one candidate tumor suppressor gene, $p16^{INK4a}$. The $p16^{INK4a}$ gene, localized to 9p21, is homozygously deleted in multiple tumor cell lines [19,20] and hemizygous deletions are observed in some lung tumors [21,22]. Recent studies also indicate that aberrant promoter region methylation may play a role in $p16^{INK4a}$ gene inactivation [23]. There was a direct correlation for $p16^{INK4a}$ gene hypermethylation and loss of $p16^{INK4a}$ gene expression in non-small-cell lung cancers. In addition, loss of heterozygosity within a region on mouse chromosome 4 syngeneic to human chromosome 9p21-22 was observed in 50% of carcinomas induced in C_3AF_1 mice by NNK [24].

The marked increase in MT activity detected by this microassay in alveolar type II cells following treatment with NNK suggests that changes in the expression of this gene could be useful as an early marker for premalignancy. A nonradioactive, rapid (1 day) in situ hybridization technique was developed to compare MT gene expression to gene activity during lung tumor progression. An antisense 24 bp oligonucleotide from the 5′ end of the murine MT gene was synthesized with 6 biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling. Strong hybridization signals were seen in hyperplasias, adenomas, and adenocarcinomas within the lungs of A/J mice (Nikula, unpublished). Studies are in progress to determine whether increased MT expression can be detected in individual type II cells prior to development of hyperplasias.

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Toxicology Letters

Physiologically based pharmacokinetic (PB-PK) models in the study of the disposition and biological effects of xenobiotics and drugs

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Abstract

Physiologically based pharmacokinetic (PB-PK) models have been used to study the mechanisms of disposition of drugs and xenobiotics for almost 70 years. Their widespread application in toxicology began 15 years ago with models for polychlorinated biphenyls and other persistent lipophilic compounds. Quantitative applications of PB-PK models in carcinogen risk assessment date to the development of a number of PB-PK models for dichloromethane in the mid 1980s. The expanding use of these models is primarily related to their ability to make more accurate predictions of target tissue dose for different exposure situations in different animal species, including humans, and to evaluate quantitatively the mechanisms of disposition of chemicals within the body. This paper discusses contemporary uses of PB-PK modeling in the context of risk assessment with xenobiotics and of safety assessment with drugs.

Keywords: Pharmacokinetics; PB-PK models; Risk assessment; Extrapolation; Dioxin; Retinoids; Safety assessment

1. Introduction

1.1. Historical perspective on the development of physiologically based pharmacokinetic (PB-PK) modeling

The systematic application of PB-PK modeling to a class of compounds began with studies of volatile anesthetics. In 1924, Haggard [1] modeled the uptake and distribution of diethylether and similar anesthetic agents using a fairly simple model that specified the role of ventilation, blood flow, and blood:air solubilities in chemical uptake into the body. Further work on PB-PK modeling with anesthetic gases was done by Kety [2], Mapleson [3], and Riggs [4]. Fiserova-Bergerova [5] extended these models to metabolized gases of occupational interest in the 1970s.

The development of PB-PK models for pharmaceuticals dates from the 1930s and the work of Teorrel in Sweden [6]. He systematically described the factors governing the disposition of a drug in the body and outlined the relevant mass-balance equations required to forecast drug concentrations over time. Unfortunately, existing computational methods only permitted the solution of these equations for very simple cases. Chemical engineers began working with these PB-PK models around 1970 and applied these physiological techniques to forecasting the disposition of various chemotherapeutic agents [7]. By

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this time there were significant improvements in computational techniques allowing these systems of mass-balance equations to be solved by numerical techniques. A review of applications of PB-PK models for drugs was published in 1983 [8].

In toxicology, PB-PK models were used by chemical engineers to study the pharmacokinetics of lipophilic chemicals, including polyhalogenated biphenyls [9], Kepone® [10], and 2,3,7,8-tetrachlordibenzofuran [11]. Toxicologists interested in chemical risk assessment then applied PB-PK modeling to certain volatile organic compounds (VOCs) that had been demonstrated to cause cancer in experimental animals. The first quantitative use of these PB-PK models in risk assessment was with methylene chloride (DCM; dichloromethane), where a PB-PK model was used to estimate tissue dose of reactive metabolites [12]. Since the work with DCM, PB-PK approaches have been used with a variety of other compounds to support dosimetry estimates in humans as part of the contemporary risk assessment process [13,14].

2. PB-PK modeling in risk assessment

Risk assessment consists of 4 parts: hazard identification, dose-response assessment, exposure assessment, and risk characterization. Hazard identification relies on animal studies and epidemiological research to identify the toxic effects of chemicals. When animal studies form the basis of the risk assessment, the relationships between exposure intensity and response is first established in animals and an acceptable exposure concentration for people is calculated based on some prescribed set of steps. This process of defining the acceptable human exposure level is part of the dose-response assessment. The prescribed steps are referred to as default methods, i.e., the steps used on a routine basis in the absence of evidence that suggests that another risk assessment approach might be more reasonable. The current default cancer dose-response assessment procedures presume linear low-dose extrapolation and apply a surface area correction for interspecies extrapolation. The surface area correction effectively assumes that humans are about 12 times more sensitive than mice or 6 times more sensitive than rats to a given daily dose (in mg/kg/day) of carcinogen. In the past, these extrapolation procedures have been used for all carcinogens regardless of their mechanisms of dosimetry or their mechanisms of toxicity.

In recent years there has been a continued effort to improve the scientific basis of both cancer and non-cancer dose-response assessments by incorporating information about pharmacokinetics – the detailed mechanisms by which chemicals are distributed from the external environment to target tissues; and pharmacodynamics – the detailed mechanisms by which target tissue doses are transformed into adverse biological responses. Insights derived from PB-PK models have been used to augment, refine or displace the current default risk assessment methods. In developing PB-PK models, a number of biological parameters are required in the animal species of interest (Fig. 1). They include: (1) physiological parameters, such as blood flows, pulmonary ventilation, organ volumes, and growth characteristics; (2) biochemical parameters, such as partition coefficients, tissue binding constants, rates of metabolism and tissue clearances; and (3) experimental parameters associated with routes and duration of exposure and timing of sampling for tissues and excreta.

3. PB-PK model-based extrapolations

Extrapolation strategies are predicated on the ability to assign causality between chemical exposure and a biological effect and to identify the form of the chemical responsible for the effect. DCM is an important solvent used in many industries. Inhalation studies in mice showed that DCM increased the incidence of cancer in the liver and in the lungs. In 1987, a risk assessment strategy was proposed for DCM based on a PB-PK model [12]. This risk assessment used biological information to infer the putative carcinogenic metabolite and a PB-PK model to

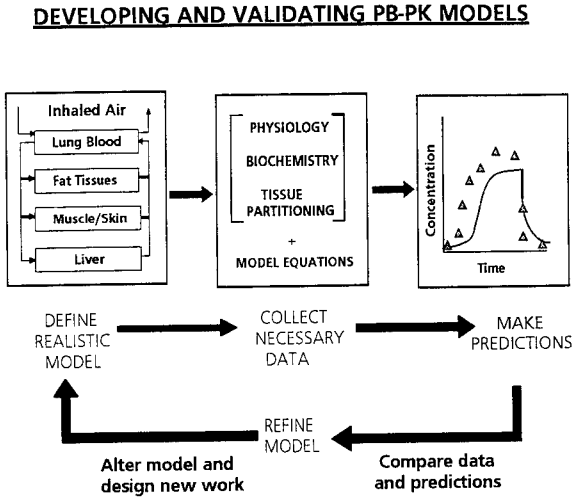


Fig. 1. An idealized schematic for PB-PK model development: the steps involved are: (1) specification of the model based on the anatomical/physiological structure of the animal; (2) parameterization of the model with physiological, physico-chemical, and biochemical constants; and (3) simulation of behavior that can be tested against actual kinetic data from the animals. If the predictions deviate significantly, changes in the model structure, consistent with physiological and biological processes, may have to be considered.

assess the tissue exposure to the metabolite under diverse exposure situations in different animal species.

The metabolic pathways and kinetic parameters of DCM biotransformation were already well established [15]. DCM is oxidized by a cytochrome P450-mediated reaction and conjugated with glutathione by a glutathione *S*-transferase (GST)-dependent pathway. Based on tumor formation dose-response data and specific studies of the mutagenicity of DCM in the presence of glutathione, DCM carcinogenicity was correlated with products of the GST pathway. Therefore, the PB-PK model was designed to account for administration by inhalation and gavage in water, GST-related DCM metabolism in target tissues (liver and lung), and species differences in PK processes.

PB-PK models provide an alternative approach to extrapolations by predicting target tissue doses at low chemical concentrations and in different species, including humans. These extrapolations depend on the manner in which physiological and biochemical parameters change between a test animal species and people. Physiological parameters change fairly coherently, but metabolic parameters are more dependent on the nature of specific enzymes in different species. Metabolic parameters need to be measured in tissues from both test animals and humans. These measurements have been made for DCM and permit development of PB-PK models for mice and humans. The estimated tissue exposure to GST pathway metabolites from the completed DCM model for mice and humans was used in a risk assessment and indicated that the default procedures overestimated the risk posed to humans from ingestion or inhalation of this solvent by 100- to 200-fold [12]. These differences were associated with non-linearities in the contribution of the GST pathway at lower doses and the lower activity of GST isoforms in human as compared to mouse tissues. Since this initial work with DCM, there has been rapid development of a number of PB-PK models for different types of toxic chemicals. The list in Table 1 from Leung [13] is already seriously dated. Continuing applications in the pharmaceutical area are discussed by Sugiyama in this workshop [16].

4. Target tissue dose

Models do not exist in a vacuum. They are developed to evaluate some problem. The PB-PK models described here are intended to evaluate the relationship between external exposure, target tissue dose, and biological outcome. A critical design criterion then is that the model actually predicts the correct measure of tissue dose. Target tissue dose has to be defined in light of our understanding of the biological mechanisms associated with toxicity or with therapeutic efficacy. Thus, development of pharmacokinetic models for risk assessment, efficacy studies, or safety assessment requires some understanding of, or presumption about, the mechanistic steps intervening between absorption of parent chemical and the expression of the relevant biological effect of the compound.

Table 1
Environmental toxicants with PB-PK models – 1991 [13]

Benzene	Kepone
2-Butoxyethanol	2-Iodo-3,7,8-trichlorodibenzo-*p*-dioxin
Carbon tetrachloride	Lead
Chloroform	Methylethylketone
Chloropentafluorobenzene	Nickel
1,2-Dichloroethane	Polychlorinated biphenyls
1,2-Dichloroethylene	Styrene
Dichloromethane	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
Dieldrin	2,3,7,8-Tetrachlorodibenzofuran
1,2-Dioxane	Tetrachloroethylene
2,2′,4,4′,5,5′-Hexachlorobiphenyl	1,1,1-Trichloroethane
Hexane	Trichloroethylene

With DCM, the measure of target tissue dose was the amount of metabolites formed from the GST conjugation pathway per day per unit volume of the target tissues. Association of this pathway with toxicity was not accomplished with pharmacokinetic studies. A variety of biological research and dose-response studies were required to generate the hypothesis of GST-related carcinogenicity. The PB-PK model integrates these observations and other pertinent information to predict exposure concentrations that are likely to be safe and those that are likely to lead to toxicity. There are many potential measures of tissue dose. Even within a class of chemicals, the relevant measure of tissue dose may vary from compound to compound. With chloroform ($CHCl_3$), oxidative metabolites are more directly related to toxicity and should be the focus of the PB-PK models [17]. Among the potential dose metrics for drugs are tissue concentrations, the integrated target tissue exposure to the drug or to active metabolite, or the extent of occupancy of critical receptors.

5. PB-PK modeling and receptor-mediated toxicity

Chemicals interact with biological targets to initiate a chain of events leading eventually to organism level effects regarded as beneficial (drugs) or deleterious to health (toxic compounds). These interactions can be associated with direct reactivity with target molecules or with the binding of the compound to a specific receptor. In the toxicology community there has been increasing interest in tailoring risk assessment approaches to compounds that interact with membrane-localized or cytoplasmic receptors. The best example of this initiative is with dioxin (TCDD; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin).

Dioxin interacts with a cellular protein aggregate consisting of the *Ah* receptor, 2 molecules of heat shock protein, and at least one other protein. The complex formed between the *Ah* receptor, dioxin, and the *Ah* receptor nuclear translocator protein (Arnt), alters transcription of some cellular proteins by binding to dioxin response elements (DREs) in promoter regions of specific genes. Among the genes transcriptionally regulated by the *Ah* receptor-dioxin complex (although not necessarily in concert with Arnt) are several that regulate cell growth and differentiation, critical processes which are altered by dioxin as part of its broad spectrum toxicity on multiple organ systems, including action as a hepatic tumor promoter. Thus, the receptor-mediated, gene-transcription mechanism of toxicity for dioxin is known in general, but many of the details are lacking, partly at least, because the biological role of the receptor and the identity of its natural ligand(s) have not been established.

Models have been developed at several different levels of detail, including empirical descrip-

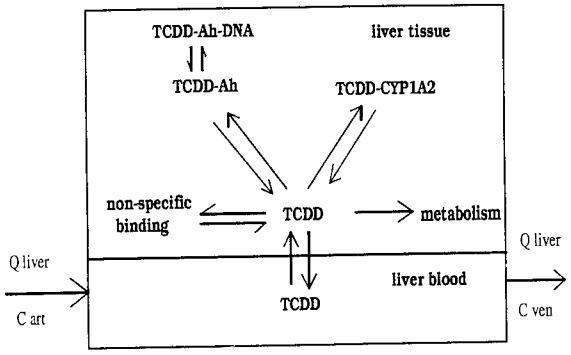


Fig. 2. Schematic of the mechanistic determinants of dioxin accumulation: dioxin accumulates in the liver due to partitioning (non-specific binding) and specific binding to both the *Ah* receptor and CYP 1A2. In addition, CYP 1A2 is under transcriptional control by the *Ah* receptor-dioxin complex, leading to non-linear effects on dioxin distribution.

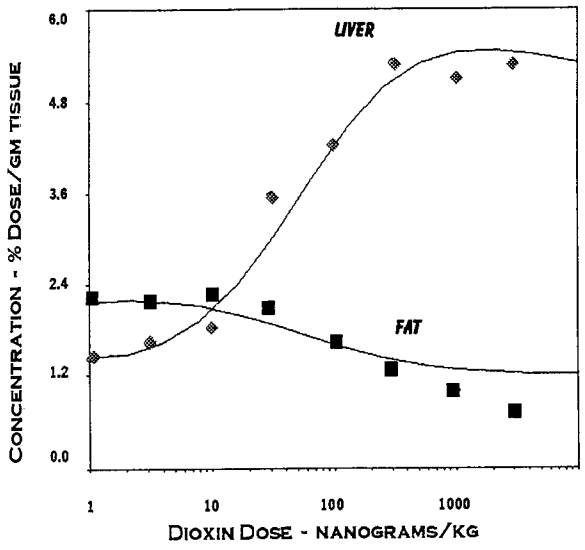


Fig. 3. Predicted relationship between the normalized liver concentration of dioxin and administered dose from a PB-PK model. Data from Abraham et al. [23] for concentrations of dioxin in liver and fat in rats 168 h after s.c. injection of various dose levels of dioxin. Smooth curves are predictions from a PB-PK model [16] that included CYP 1A2 induction. The placement of curves along the dose axis and the overall shape of these curves permits inferences about the binding affinities and capacities of dioxin for the *Ah* receptor and CYP 1A2. The accuracy of the inferences, of course, depends on the fidelity of the model with the actual biology.

tions that look at distributional, non-linearities from a physiological perspective [18], physiological models that capture the solubility characteristics of dioxin [19], and the more comprehensive PB-PK models that consider induction of several gene products [20–22]. One of the proteins regulated by the dioxin-*Ah* receptor complex binds dioxin. This protein is believed to be cytochrome P450 1A2 (CYP 1A2). PB-PK models accounting for dose-dependent distribution of dioxin between liver and fat have to account for the time-dependent changes in CYP 1A2 due to induction. Thus, the tissue compartments in the PB-PK model (Fig. 2) for dioxin have to include gene induction, a pharmacodynamic process. Quantitative PB-PK/gene induction models for dioxin must express this induction in reasonable biological detail and in mathematical formulae that can predict the extent of induction at various dioxin doses and in various species.

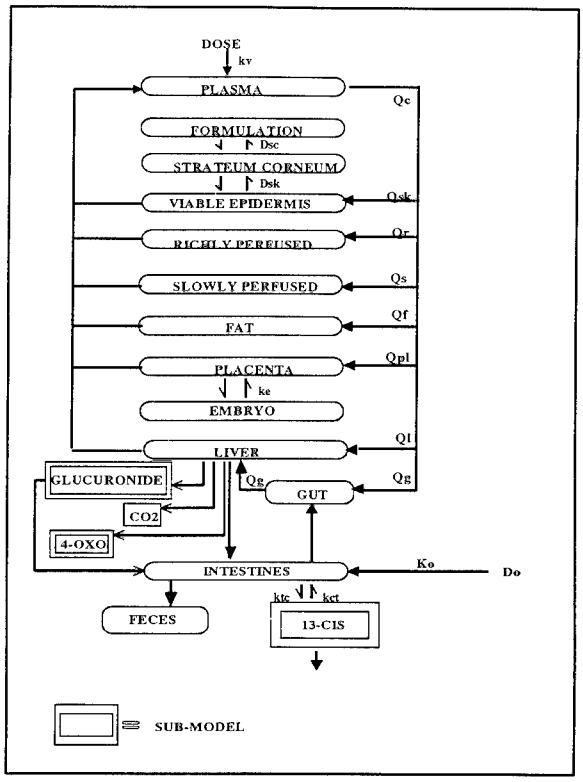
In the induction models dioxin first binds to the *Ah* receptor and this dioxin-*Ah* complex then binds to DREs. The equations for the rate of change of the amount of CYP 1A2 in the liver include synthesis rates and degradation rates of the binding protein. Induction is modeled as an increase in the rate of synthesis, consistent with transcriptional activation, that increases to a maximum when all DRE promotional sites are occupied. The increase in rate of synthesis depends on the concentration of the *Ah*-dioxin complex according to the DRE-*Ah* complex dissociation constant and a Hill-coefficient for binding of the complex to the DNA-DRE sites. This CYP 1A2 induction model is substantially simplified compared to known biological processes. The rate constants and binding constants in these gene induction models have been described phenomenologically. They are estimated by fitting the models to time-course or dose-response observations of tissue dioxin concentrations and the amounts of inducible enzyme in the liver at various times after acute or chronic dosing with dioxin. The fit to data from Abraham et al. [23] appears in Fig. 3. While individual rate constants in this model are difficult to interpret in precise biological terms, these models permit

reconstruction of diverse dose-response data based on a small number of fitting constants and have been helpful in suggesting new risk assessment-oriented research directions [20]. Even though these PB-PK and physiologically based pharmacodynamic (PB-PD) models will probably never be completely isomorphic with the detailed behavior of the biological organisms, they remain the best available tools for improving the biological basis of the 2 essential components of dose-response assessments, i.e., the low-dose and interspecies extrapolations.

6. Retinoids

High doses of all-*trans*-retinoic acid (ATRA) and 13-*cis*-retinoic acid (13-cis) are teratogenic in various animal species. The active compounds appear to be these acids and their oxo-derivatives. Since isomerization between forms occurs in vivo, all 4 active chemical species are present in animals and people after dosing with either of the parent compounds. The pharmacokinetics of ATRA have been examined in several animal species and one of the challenges with these retinoids is the complexity of metabolic pathways for these endogenous growth regulatory chemicals. With the VOCs, there are generally only 1 or 2 dominant biotransformation pathways. With TCDD, the toxicity is associated with altered gene expression due to the activity of the *Ah* receptor-dioxin complex. Metabolism serves to reduce the concentration of the toxic chemical and the TCDD metabolites do not have significant toxicity themselves. With the retinoids, however, the task faced in PB-PK model development is estimation of the concentration of all possible teratogenic species under different therapeutic situations.

We have developed a PB-PK model for aiding in safety assessment of the teratogenic risks posed by retinoid exposure and applied to a wide variety of literature data (H.J. Clewell III and M.E. Andersen, work in preparation). The model elements (Fig. 4) include portals of entry (gut, skin, and blood), target tissues (embryo/fetus), and the compartments essential for describing



A PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR ALL-TRANS-RETINOIC ACID

Fig. 4. A schematic for a dermal uptake PB-PK model for ATRA. The model has to account for biliary excretion of glucuronides, intestinal hydrolysis of the glucuronide to ATRA, and ATRA reabsorption. Interconversion of *cis*- and *trans*-isomers occurs in the intestine. Equivalent PB-PK models have to be developed for chemicals in double-lined boxes and for 13-*cis* glucuronide and 13-*cis*-4-oxo and its glucuronide. The target site is the embryo and the tissue dose metric is total embryo exposure to the 4 active retinoids.

storage, biotransformation, and the interconversion between *trans*- and *cis*-isomers that take place in biological systems. Compared to the DCM or TCDD models, the retinoid PB-PK model contains considerably more equations and assumptions about the metabolic pathways of these retinoids and require modeling of at least 4 potentially active teratogens. Nonetheless, the approach in developing the model is the same as with any other model development problem for biologically active compounds; i.e., (1) identify

the determinants of disposition and biotransformation; (2) develop the mathematical description of the biological processes involved; (3) test the model against available data; (4) identify new experiments to test the robustness of the model description; and (5) collect the necessary experimental data to confirm or refute the model structure (Fig. 1). The final model output in this example with ATRA is total fetal exposure to potentially teratogenic retinoids.

7. Summary/recommendations

PB-PK models will play a larger and larger role in integrating diverse toxicological/pharmacological data within a biologically realistic framework, in establishing dose-response extrapolations for important environmental contaminants, and in designing specific research to improve safety and risk assessments with important drugs and xenobiotics. In developing these models it is important that they address the appropriate question. The issue is not simply to develop elegant PB-PK models; it is to develop models of appropriate measures of tissue dose and to ascertain how these models should be used to infer low-dose and interspecies effects on toxicity, safety, and efficacy. The models need to be formulated based on the best contemporary ideas of the manner in which dose to tissues leads to critical cellular alterations affecting biological outcome.

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Applications and prospects for physiologically based pharmacokinetic (PB-PK) models involving pharmaceutical agents

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Abstract

Because of the increasing availability of human liver samples, we are now able to predict in vivo drug disposition in man from in vitro metabolic and binding studies. In this report, we summarize successful attempts to predict in vivo metabolic clearances in animals and humans from in vitro biochemical parameters, using physiologically based pharmacokinetic models. There are still some problems, however, in extrapolating in vivo hepatic metabolism in man from in vitro data obtained using human liver specimens, due to (1) large interindividual differences resulting from genetic polymorphism and/or (2) differences in enzyme activities depending upon the conditions under which liver specimens may have been kept. We propose a possible method to overcome these difficulties by applying the concept of a 'scaling factor'. In addition, we also review several additional factors which should be considered to help achieve more reliable predictions.

Keywords: Drug metabolism; In vitro/in vivo scaling; Physiologically based pharmacokinetic model

1. Introduction

The success of interspecies scaling of biochemical parameters such as tissue binding and metabolism will enable us to predict successfully drug disposition in man [1,2]. Several attempts have been made to scale the intrinsic parameters representing unbound intrinsic clearance (CL_{int}) and distribution volume of unbound drugs taking into account allometric considerations based on body weight [1,2]. Although such an allometric approach allows the prediction of the pharmacokinetics of a number of drugs in man, there are some cases where the data do not fit the extrapolations, bearing in mind that this scaling approach is empirical.

On the other hand, much progress has been made in correlating metabolic clearance, estimated from in vitro studies using isolated hepatocytes or subcellular fractions such as microsomes and $9000 \times g$ supernatants, with unbound intrinsic clearances observed in vivo ($CL_{int,\ in\ vivo}$) [3,4]. It is important to examine this approach, since it might be an alternative way of predicting in vivo drug disposition in man, in view of the increasing availability of human liver. From this standpoint, we wish to review the attempts to scale in vivo drug disposition in

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experimental animals and humans from in vitro data and then discuss some important factors which need to be considered.

2. Prediction of in vivo hepatic clearance from in vitro data

2.1. Hepatic metabolism in rats

Kinetic parameters for enzymes (such as K_m, V_{max} and $CL_{int,\ in\ vitro}$ $(=V_{max}/K_m)$) have been estimated from in vitro studies using isolated hepatocytes or subcellular fractions such as microsomes. These parameters can be converted into values for the whole organ with a knowledge of the enzyme mass recovery for the preparation used. Combination of these $CL_{int,\ in\ vitro}$ values for unbound drugs, unbound fraction in the blood (f_B) and hepatic blood flow (Q_H) can give hepatic clearances (CL_H), hepatic extraction ratios (E_H) and hepatic availability (F_H) based on appropriate organ perfusion models. These estimated parameters can then be easily compared with those obtained from in vivo studies or liver perfusion experiments [3,4]. Using this approach, attempts to predict hepatic extraction from in vitro data have been successful for many drugs [5,6]. Several kinetic models to describe the hepatic elimination of drugs have been used to relate CL_H to Q_H, f_B and CL_{int} [7,8].

The predicted values depend upon the models selected particularly for highly cleared drugs. The venous equilibrium and sinusoidal perfusion models have been most frequently used, mainly due to the relatively simple mathematics involved [7,8]. Although the in vitro data have generally given good predictions, the F_H of drugs in vivo or in perfused liver systems seems to be smaller or larger than that expected from in vitro experiments based on the venous equilibrium model and sinusoidal perfusion model, respectively [3,9]. The distributed and dispersion models [10,11] gave better predictions of in vivo metabolism from in vitro experimental data, even for high clearance drugs (Fig. 1) [5,6].

2.2. Human hepatic metabolism

Some successful attempts to apply the in vitro/in vivo scaling method to humans have been published [12,13]. Bayliss et al. [12] determined the metabolic rate of loxtidine, an H_2-blocker, using hepatocytes isolated from rats, dogs and humans. The parameters obtained were converted to values per g liver based on the recovery mass of the number of hepatocytes. In addition, the in vivo CL_H was estimated by subtracting the renal clearance from the total body clearance (CL_{tot}) obtained from data following i.v. ad-

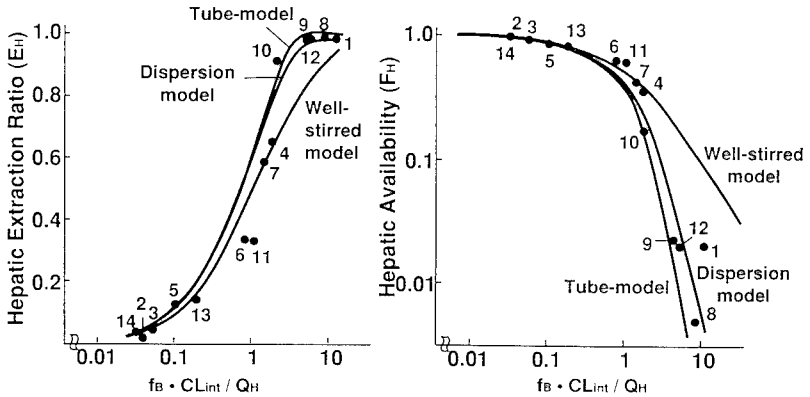


Fig. 1. Prediction of hepatic extraction ratio (E_H) and hepatic availability (F_H) from in vitro measurement of intrinsic metabolic clearance in rats. (1) Alprenolol; (2) antipyrine; (3) carbamazepine; (4) diazepam; (5) ethoxybenzamide; (6) hexobarbital; (7) 5-hydroxytryptamine; (8) lignocaine; (9) pethidine; (10) phenacetin; (11) phenytoin; (12) propranolol; (13) thiopental; (14) tolbutamide. (From Y. Sugiyama et al. (1993) In: Methods and Techniques in Studying Pharmacokinetics: From Preclinical Study to Phase-I Study. ed. by Y. Sugiyama (Japanese Association for Xenobiotics Metabolism and Disposition), pp. 87–108).

ministration of the drug. Then, the $CL_{int, in\ vivo}$ was calculated from CL_H in vivo based on the well-stirred and dispersion models taking into consideration the Q_H of each animal species. $CL_{int, in\ vitro}$ estimated from the in vitro data was comparable with $CL_{int, in\ vivo}$. Since loxtidine exhibits a low E_H (approximately 0.3 in rats), no marked difference was observed in the E_H between the 2 models.

We also attempted to apply the in vitro/in vivo scaling method to humans, based on previously reported parameters on in vitro metabolism and plasma protein binding and on in vivo drug disposition for 25 different metabolites [5]. The $CL_{int, in\ vitro}$ values obtained from the in vitro metabolic studies were converted to values per g liver, as described previously [5]. Similarly, $CL_{int, in\ vivo}$ was calculated from CL_H in vivo using the dispersion model [5]. For 20 metabolites, $CL_{int, in\ vitro}$ were comparable with $CL_{int, in\ vivo}$ (Fig. 2) [5]. Although the $CL_{int, in\ vitro}$ generally exhibited a positive correlation with $CL_{int, in\ vivo}$, a large difference (several-fold) was observed in CL_{int} for some drugs when comparing in vitro with in vivo (Fig. 2) [5]. This may be at least partly due to extrinsic variabilities in enzyme activity arising from the conditions under which the livers had been removed and stored.

In addition, several recent reports indicate the presence of poor metabolizers who lack specific cytchrome P450 isozymes (polymorphic metabolism). It may be important to identify the content of each P450 isozyme present in liver specimens if the isolated hepatocytes or subcellular fractions are to be used for in vitro metabolic studies in humans.

2.3. Application of the scaling factor

It may thus be possible to predict hepatic metabolism in vivo from in vitro clearance data if the metabolic rate in vitro can be determined under the appropriate experimental conditions. It may be difficult, however, to determine the appropriate experimental conditions by using the relatively limited amounts of such valuable material as human liver. For this reason, we would like to propose an approach [5,6].

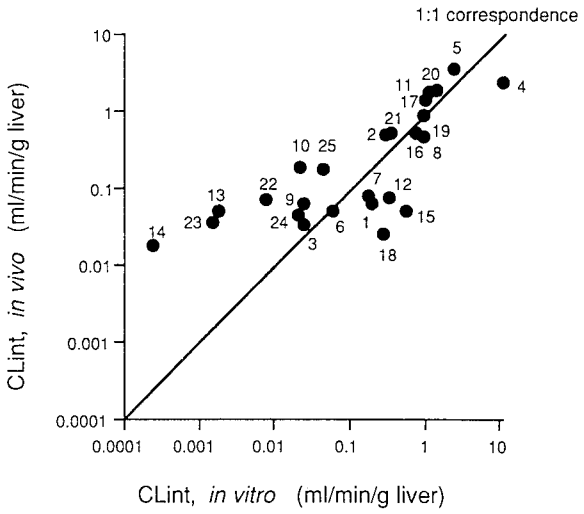


Fig. 2. Comparison of $CL_{int, in\ vitro}$ with $CL_{int, in\ vivo}$. Previously reported parameters on in vitro metabolism and plasma protein binding and on in vivo drug disposition for 25 different metabolites were analyzed to determine $CL_{int, in\ vitro}$ and $CL_{int, in\ vivo}$. (1) Alprazolam; (2) diazepam; (3) dofetilide; (4) imipramine; (5) lidocaine; (6) loxitidine; (7) α-hydroxymetoprolol; (8) *O*-demethylmetoprolol; (9) hydroxymethylmexiletine; (10) *p*-hydroxymexiletine; (11) phenacetin; (12) quinidine; (13) 1,3-dimethyluric acid + 1-methylxanthine; (14) 3-methylxanthine; (15) tolbutamide; (16) norverapamil (R); (17) D-617 (R); (18) D-703 (R); (19) norverapamil (S); (20) D-617 (S); (21) D-703 (S); (22) 6-hydroxywarfarin (R); (23) 7-hydroxywarfarin (R); (24) 6-hydroxywarfarin (S); (25) 7-hydroxywarfarin (S). From [5].

In this approach, reference compounds are used which satisfy the following 2 criteria [5,6]. (1) The reference compound should be metabolized by the same enzyme as the drug of interest; (2) the disposition of the reference compound is already documented. The initial velocity of metabolism obtained for the drug is divided by the drug concentration in the incubation medium to give the $CL_{int, in\ vitro}$. It might be difficult to compare directly $CL_{int, in\ vitro}$ values with $CL_{int, in\ vivo}$, since the initial velocity should be determined under appropriate conditions (i.e., adjustment of concentrations of co-factor(s), metal ions and oxygen). Then, the scaling factor by which the values obtained in in vitro studies are converted to in vivo values can be estimated as follows. Firstly, CL_H for some reference compounds are calculated from in vivo pharma-

cokinetic data. Taking into account the Q_H and the f_B, the aforementioned CL_H can be converted to a $CL_{int,\ in\ vivo}$ for each reference compound using the appropriate pharmacokinetic model, e.g. the dispersion model. The ratio of $CL_{int,\ in\ vivo}$ to $CL_{int,\ in\ vitro}$ is defined as a scaling factor. If the scaling factor obtained for each reference compound is comparable, we can apply this to the in vitro/in vivo scaling for the intrinsic clearance of the drug of interest [5,6].

2.4. Drug-drug interaction

Based on the approach described previously, we can predict in vivo drug-drug interactions from in vitro metabolic and binding studies [5,6,14]. In this section, previous studies from our laboratory on the interaction between tolbutamide (TB) and sulfonamides (SAs) [15–17] are described, preceded by a general discussion.

2.4.1. General discussion

Drug-drug interactions can be predicted quantitatively based on in vitro metabolic and binding studies. If we assume that the simultaneously administered drug inhibits the metabolism of the study drug in a competitive manner, the intrinsic clearance for metabolism is given by:

$$CL_{int} = \frac{V_{max} \times C_{u.h}}{K_m\ (1 + I_{u.h}/K_i) + C_{u.h}}$$

where $C_{u.h}$ and $I_{u.h}$ represent the hepatic unbound concentration of the study drug and the simultaneously administered drug, respectively. If (1) the kinetic parameters for the metabolism (K_m, K_i and V_{max}) are determined in vitro and (2) the time profiles for $I_{u.h}$ are determined, we can predict the alterations in the in vivo metabolic clearance according to the method described previously; the plasma unbound concentration of simultaneously administered drug can be substituted for $I_{u.h}$. We must be cautious in making the prediction, however, if the extensive metabolism of the second drug is observed; for such drugs, the plasma unbound concentration exceeds $I_{u.h}$. Furthermore, a satisfactory prediction based on this method may not be obtained if the second drug is taken up by the liver via an active transport process; for such drugs, the plasma unbound concentration underestimates $I_{u.h}$.

2.4.2. TB-SA interaction

The drug interaction between TB and a second drug has been studied extensively [14], since severe hypoglycemia in diabetic patients has been observed after the simultaneous administration of TB and sulfaphenazole (SP). Furthermore, species differences have been reported in the TB-SP interaction; in rats and humans, SP markedly prolonged the plasma half-life of TB, whereas SP accelerated the disappearance of TB in rabbits. Since TB is a low-clearance drug whose CL_{tot} can be approximated by f_B multiplied by CL_{int}, the effect of SAs on the f_B and CL_{int} of TB was examined in both rats and rabbits.

In vivo, TB was administered as a bolus to rats and rabbits whose plasma SP concentrations were kept constant throughout the experiments by i.v. infusion [15–17]. N^4-acetyl-SP, a metabolite of SP, was also detectable in rabbit plasma. In rats, the CL_{tot} of TB was markedly reduced by SP treatment, resulting in a prolonged TB half-life [15–17]. In contrast, the CL_{tot} of TB was slightly increased (15–30%) by SP treatment in rabbits [15–17]. The following in vitro experiments were performed to clarify the mechanisms behind these observations.

In rats, SP competitively inhibited the plasma protein binding of TB [16]. At in vivo concentrations, SP increased the f_B value of TB approximately 2-fold (Fig. 3) [15,16]. In the same manner, the f_B of TB in rabbits was increased approximately 2-fold in the presence of SP and N^4-acetyl-SP at their in vivo concentrations [17], indicating that the species difference observed in vivo cannot be accounted for only by alterations in the plasma protein binding of TB (Fig. 3) [15–17]. Competitive inhibition of TB hydroxylation by SP and N^4-acetyl-SP was also observed in in vitro experiments using liver microsomes [15–17]. Based on the kinetic parameters for the metabolism of TB determined in vitro, the alterations in $CL_{int,\ in\ vitro}$ induced by SP treatment could be predicted at the in vivo mean plasma concentration. Although a moderate decrease in $CL_{int,\ in\ vitro}$ produced by SP treatment was suggested in rabbits, the decrease was much more marked in rats (Fig. 3). The in vitro $CL_{tot,pred}$ defined by f_B multiplied by $CL_{int,\ in\ vitro}$ thus

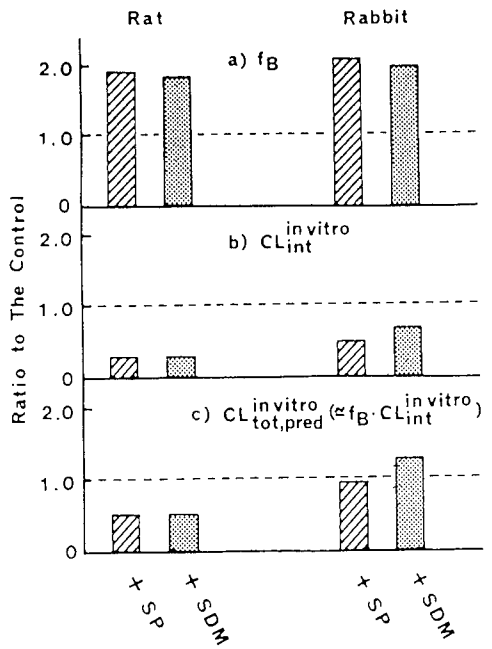


Fig. 3. Ratios of f_B, $CL_{int,\ in\ vitro}$, and $CL_{tot,pred}$ of TB in the presence of SA to those in the absence of SA. These ratios were calculated based on the in vitro experiments. The dotted line in each panel shows the ratio of 1.0. (a) f_B; (b) $CL_{int,\ in\ vitro}$; (c), $CL_{tot,pred}$. SDM, sulfadimetoxin. From [9].

showed a marked species difference; the net change in in vitro $CL_{tot,pred}$ induced by SP was minimized in rabbits due to the compensatory changes in f_B and $CL_{int,\ in\ vitro}$ (Fig. 3) [15–17]. In contrast, the decrease in $CL_{int,\ in\ vitro}$ overcame the increase in f_B in rats [15,16], resulting in a decrease in in vitro $CL_{tot,pred}$ (Fig. 3). In the same manner, the species difference in TB-sulfadimethoxine interaction was accounted for by considering the alterations in f_B and $CL_{int,\ in\ vitro}$ of TB determined in vitro (Fig. 3). Collectively, we were thus able to clarify the mechanism for the in vivo drug-drug interaction based on in vitro experiments [15–17].

3. Other factors which should be considered in performing in vitro/in vivo scaling

The previously described in vitro/in vivo scaling should be possible if we can assume (1) a rapid equilibrium between blood and hepatocytes; (2) availability of only the unbound drug for elimination and transport across membranes; and (3) homogeneous distribution of metabolic enzymes and transport carriers along the blood flow pathways in the liver. Recent reports indicate that these assumptions do not always hold [2]. In the final part of this article, we will discuss these factors.

3.1. Estimation of membrane permeability

In some instances, the CL_H of a drug which is completely eliminated by metabolism may not simply be a function of only Q_H, f_B and CL_{int}; the clearance for the transport across the sinusoidal membrane can affect the overall intrinsic clearance ($CL_{int,all}$) [18,19]. This relationship can be expressed by the equation: $CL_{int,all} = PS_{inf} \times CL_{int}/(PS_{eff} + CL_{int})$, where PS_{inf} and PS_{eff} are the clearance for influx and efflux, respectively, and CL_{int} is the intrinsic metabolic (or sequential) clearance [18,19].

A multiple indicator dilution technique developed by Goresky and Nadeau and by Miyauchi et al. [20,21] has the advantage that the clearance for intrinsic metabolism and for membrane penetration can be estimated. For compounds whose penetration across the sinusoidal membrane is very rapid, the effect of the putative unstirred water layer in Disse's space should be considered [22].

3.2. Heterogeneous distribution of metabolic enzymes

Although it has been assumed that the drug metabolizing enzymes are evenly distributed along the blood flow pathways in the liver, recent biochemical and kinetic studies suggest that this assumption does not necessarily hold [5,6,23,24]. For example, it has been found that cytochrome P450s are abundant on the peri-central side, while sulfate conjugating enzymes are abundant on the opposite (peri-portal) side [23–25]. It has been demonstrated by Pang [25] that this is important when examining the kinetics of metabolites. In contrast, it has been suggested that the hepatic clearance of parent drugs is unaffected by the heterogeneous distribution of enzymes if the total amount of enzymes is kept constant, assuming rapid equilibrium between blood and hepatocytes [11]. However, we have demonstrated that the extraction ratios of parent drugs can

be affected by uneven distribution of enzymes in the presence of a diffusional barrier [26].

3.3. Protein-mediated hepatic uptake

It has been reported that the uptake clearance for compounds which are highly (more than 95%) bound to plasma protein(s) is much higher than that expected based on the hypothesis that only unbound drug is available for hepatic uptake; this suggests the presence of 'albumin-mediated uptake' [5,6,27–29]. Furthermore, γ-globulin has been shown to mediate the hepatic uptake of rose bengal, indicating that the 'albumin receptor' is not necessary for this phenomenon [28]. This hypothesis is supported by the fact that albumin-mediated uptake by the perfused rat liver has also been observed in Nagase analbuminemic rats with a genetically controlled serum albumin deficiency [29]. Although the exact mechanism of 'albumin-mediated uptake' is still controversial, the most likely explanation involves the effect of the unstirred water layer in the extracellular space on the drug diffusion process [30,31].

In vitro/in vivo scaling in humans may become more reliable in the near future if we make use of these factors and are able to develop valid in vitro methods for quantifying membrane permeability as well as metabolic activity using human hepatocytes or subcellular fractions.

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Toxicokinetic models for volatile industrial chemicals and reactive metabolites

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Abstract

Two approaches of compartmental toxicokinetic modeling of gaseous compounds are presented which are suitable for kinetic analysis of concentration-time data measured in the air of closed exposure systems. The first approach is based on a two-compartment model with physiological gas uptake, the second on a physiologically-based toxicokinetic model. Both models can be used for the description of inhalation, accumulation, exhalation and metabolism of gaseous compounds together with the toxicokinetics of metabolites. Interspecies extrapolation is based on physicochemical, physiological and biochemical parameters. The advantage of the two-compartment model is its limited number of variables and its experimentally easy applicability. Its disadvantage is the impossibility to predict tissue specific concentrations. The advantage of the physiologically-based model is its usability for predictions and for the description of tissue specific concentrations. However, it entails great effort, since a series of parameters has to be determined before meaningful model calculations can be carried out.

Keywords: Toxicokinetic two-compartment model with physiological gas uptake; Physiologically-based toxicokinetic model for gas uptake studies; Closed chamber technique; Inhalation toxicokinetics; Interspecies extrapolation

1. Introduction

Toxic or carcinogenic effects of chemicals taken up by the organism may result from the compounds themselves or from metabolites thereof. The burden of the ultimate active species in different organs and tissues is determined by its concentration and the time it remains at the site of action. Such concentration-time courses of compounds and metabolites depend on the rates of absorption into and distribution between blood and tissues, on the rates of transport by the bloodstream, of biotransformation and of excretion from the body. Biotransformation can lead to active metabolites or to products of reduced effectiveness. Mostly, biotransformation processes are catalyzed enzymatically and therefore subject to saturation kinetics (e.g. according to Michaelis and Menten). Consequently, the target dose of the active

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chemical species is often not a linear function of the amount of xenobiotic taken up. In toxicological inhalation studies, different concentrations of the test compound are administered to groups of animals in order to establish a relation between the exposure concentration and the resulting toxic effect. To understand and extrapolate the relationships obtained to low concentrations at which human exposure occurs, knowledge of the burden of the ultimate active substance in both species is indispensable as a basis for risk assessment. To gain this information is one of the main tasks of toxicokinetics. Several theoretical approaches have been developed for that purpose, starting at the end of the last century with the studies of inert gases by Zuntz and later by Widmark and Haggard, which are summarized in the review of Kety [1]. Nowadays, the most widely used toxicokinetic models are compartmental models, developed on the basis of the classical works of Teorell [2,3] and, increasingly, physiological models. The advantages of the latter models for studying inhalation toxicokinetics, especially with respect to saturable metabolism and, further, to risk assessment, were demonstrated and discussed particularly by Andersen and colleagues [4,5].

In the following, a two-compartment model with physiological gas uptake, which has been developed by us for the study of the toxicokinetics of a series of gaseous compounds [6], will be compared with physiological toxicokinetic models for gas uptake studies, exemplified by a model for styrene together with its reactive metabolic intermediate, styrene-7,8-oxide [7].

2. Concepts of modeling

The determination of toxicokinetic parameters of gaseous compounds is usually based on measurements of the time course of the compound's concentrations in body fluids or in breathing air of exposed animals or humans [8]. Inhalation experiments can be carried out with constant exposure concentrations using dynamic flow-through systems or with decreasing concentrations in closed exposure chambers into which a

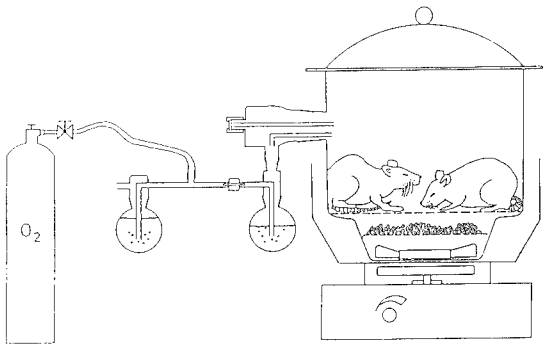


Fig. 1. Closed all-glass system for exposing laboratory animals to gaseous compounds.

defined amount of the test compound is given only once at the start of the experiment (Fig. 1). The latter method has two main advantages over the former one: first, data from a wide concentration range can be collected within a relatively short period of time; second, the amounts of the xenobiotic taken up are well below those when open systems are used. Two model approaches have been developed allowing the generation of concentration-time curves which can be fitted through the measured data in order to gain compound specific toxicokinetic parameters describing the kinetic behaviour within the organism. These models are the two-compartment model as first presented in 1979 by Filser and Bolt [9] (for a revised version containing a detailed description see [6]) and the 'physiologically-based pharmacokinetic (pbpk) model' developed by Andersen and colleagues [10]. Both model structures contain compartments which are defined by their volumes and their actual compound concentrations. In every compartment, the compound is homogeneously distributed at any time point from entering up to leaving it.

The shapes of the concentration-time courses obtained in the atmosphere of the closed exposure chambers depend strongly on the physicochemical properties of the test compound as tissue/air partition coefficients, on biochemical parameters such as the capacity and the substrate affinity of the metabolising enzymes reflected by the maximum rate of metabolism (V_{max}) together with the apparent Michaelis-constant (Km_{app}),

and on physiological processes such as alveolar ventilation and the blood flow through organs and tissues.

3. Two-compartment model with physiological gas uptake

The toxicokinetic parameters for inhalative uptake, accumulation, and elimination via metabolism and exhalation can be gained by using the 'Closed Chamber Technique (CCT)' [6]. This technique includes the measurement of gas concentrations in the closed chamber and the use of a two-compartment model (Fig. 2) for the kinetic interpretation of the data obtained. Compartment 1, which is defined by its volume V_1 and the actual air concentration y_1 of the compound, represents the atmosphere of the closed system. Compartment 2 represents the sum of the bodies of all exposed individuals having a body volume of V_2. The actual average body concentration is given by y_2. Both compartments are separated by a surface, in most cases representing the lung. Only a small number of constants have to be determined from curve fitting, which can be done by means of the program SOLVEKIN [11]. It is based on the method of the least error squares using the 'simplex' algorithm of Nelder and Mead for function minimization. The rate constants for inhalation and exhalation are described as k_{12} and k_{21}, respectively. The product of k_{12} with V_1, defined as 'clearance of uptake' is independent of the chamber's volume, since k_{12} is inversely proportional to V_1. Therefore, the value obtained is also valid for an open atmosphere with an infinitely large volume.

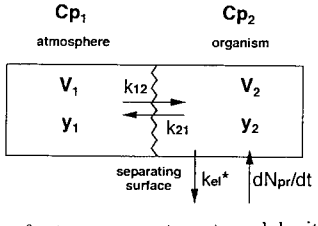


Fig. 2. Scheme of a two-compartment model with physiological gas uptake [6]. For explanation of the symbols, see text. If metabolic elimination shows saturation kinetics according to Michaelis and Menten, k_{el}^* can be expressed as $k_{el}^* = V_{max}/[V_2(Km_{app} + y_2)]$ with V_{max} being the maximum rate of metabolism and Km_{app} the apparent Michaelis constant.

The clearance of uptake multiplied by y_1 is identical to the maximal rate of inhalative uptake of the gaseous compound from air via the lung into the blood stream. In physiological terms, this rate is dependent only on the alveolar ventilation, the cardiac output, the blood/air partition coefficient of the compound, and its air concentration. The rate is related to the first inspiration breath when the capillary alveolar blood is still free of compound [12].

The 'clearance of exhalation', represented by the product of k_{21} with V_2 is a measure of the amounts of compound exhaled unchanged. The ratio of $k_{12}V_1$ to $k_{21}V_2$ equals the thermodynamic partition coefficient ('K_{eq}') whole body/atmosphere which can be reached if no metabolism occurs or at very high exposure concentrations, when metabolism is saturated. K_{eq} is identical to the sum of the products of all individual tissue/air partition coefficients with the corresponding tissue volumes divided by the whole body volume. Some compounds are produced endogenously. This is reflected by the endogenous production rate (dNpr/dt). Metabolism is taken into consideration by the introduction of k_{el}^*. Its value is constant only if the rate of metabolism is directly proportional to the average concentration in the body (linear kinetics). In case of saturation kinetics, k_{el}^* is concentration-dependent becoming continuously smaller with increasing exposure concentration. It can be used for the description of kinetics according to Michaelis–Menten, allowing the calculation of V_{max} and Km_{app}, the latter giving the average body concentration at which $V_{max}/2$ is reached. The average concentration of the compound in the organism is the result of the amounts inhaled, endogenously produced, and eliminated via exhalation and metabolism. If exposure takes place at constant atmospheric concentrations as may occur under open exposure conditions, the resulting average concentrations in the body at steady state can be expressed as the product of the atmospheric exposure concentrations and a concentration dependent bioaccumulation factor K_{st}^*. Division of Km_{app} by K_{st}^* yields the

atmospheric exposure concentration at which $V_{max}/2$ is reached. K_{st}* is a measure of the accumulation of the compound in the organism at steady state exposure. For such conditions of exposure, the accumulation in tissues can be calculated from K_{st}*, provided that the tissue/air partition coefficients are known (in prep.). Several methods are published on the determination of tissue/air partition coefficients [8,13–16].

The product of k_{el}*, V_2 and K_{st}* is defined as 'clearance of metabolism, related to the atmospheric concentration', since, if multiplied by a defined, constant atmospheric concentration, it yields the corresponding rate of metabolism at steady state. This rate is obtained, too, from the product of the alveolar ventilation (V_{alv}), the alveolar retention (R_a) at steady state and the atmospheric concentration of the compound. Consequently, the product of V_{alv} with R_a equals $k_{el}* \cdot V_2 \cdot K_{st}*$. For further definitions and explanations, see Ref. [17]. The parameters, calculated by means of the two-compartment model, can directly be incorporated into physiological models, yielding a link between both toxicokinetic approaches (in prep.). Since $k_{12}V_1$, K_{eq}, and $k_{21}V_2$ are composed of physiological and physicochemical parameters, an interspecies extrapolation of these parameters can be performed without the necessity of using assumptions which cannot be proved. Solely the extrapolation of the biochemically derived constants V_{max} and Km_{app} requires procedures such as the allometrical one which are not directly verifiable [17].

Fig. 3 depicts concentration-time courses for styrene vapors in closed exposure systems occupied by two rats in each of the experiments which are specified by the decrease of atmospheric styrene following an initial administration of a defined amount into the chamber's atmosphere. Three phases are distinguishable in the semilogarithmic plot. During the first phase, atmospheric styrene decreases rapidly due to accumulation in the exposed organism; the second together with the third phase, represented by a downward curve with a final linear part are

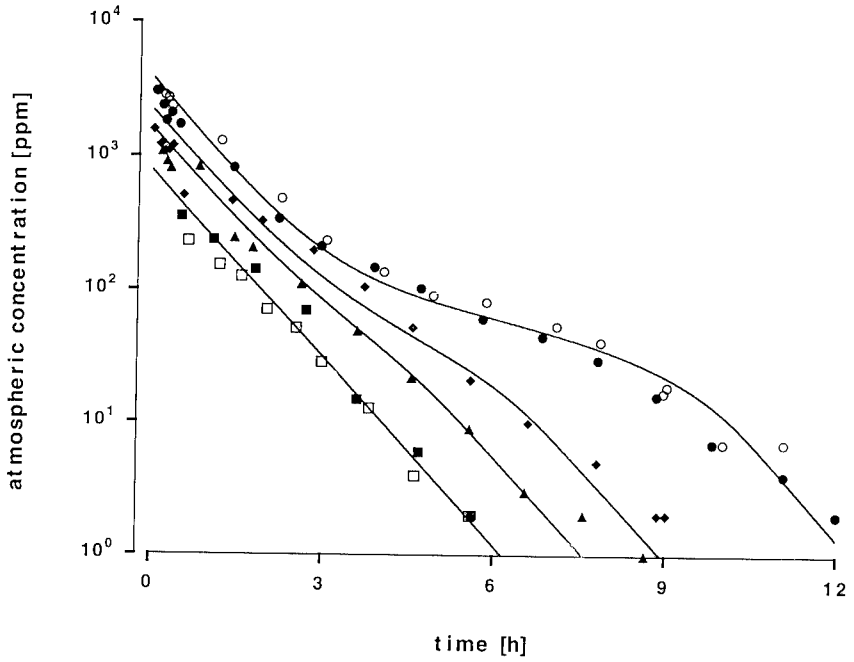


Fig. 3. Concentration-time courses of atmospheric styrene at various initial concentrations in a closed exposure chamber (6.4 l) containing two Sprague–Dawley rats in each experiment. Symbols represent measured data; solid lines were calculated by a two-compartment model [17].

characteristic for saturation kinetics. The solid lines are model fits from which the values of the toxicokinetic constants k_{12}, k_{21}, V_{max} and Km_{app} are derived (Fig. 2). A similar inhalation study was carried out with mice [17]. The values obtained for Km_{app} and for the parameters $k_{12}V_1$, $k_{21}V_2$ and V_{max} calculated for one rat and one mouse, respectively, were used to construct the plots given in Fig. 4. For steady state exposures to constant styrene vapor concentrations, they show the concentration dependences of K_{st}*, of the average styrene concentration in the body, of the rate of metabolism and of the ratio of exhaled styrene to inhaled styrene. Qualitatively, mice and rats behave similarly when exposed to styrene, however there are quantitative differences. The influence of the saturation of the metabolism on the kinetic parameters is obvious. Below about 300 ppm, K_{st}* is very small compared to K_{eq}, and styrene accumulates only little within the organism; it is very efficiently metabolized, the rate of metabolism being proportional to the atmospheric styrene concentration. Only small amounts are exhaled as the unchanged compound. With increasing concentrations, K_{st}* approaches the thermodynamic partition coefficient K_{eq} and consequently styrene accumulates to much higher levels in the organism. Since metabolism becomes saturated, exhalation of unchanged styrene becomes the main route of elimination. A species extrapolation of these results to the human exposure

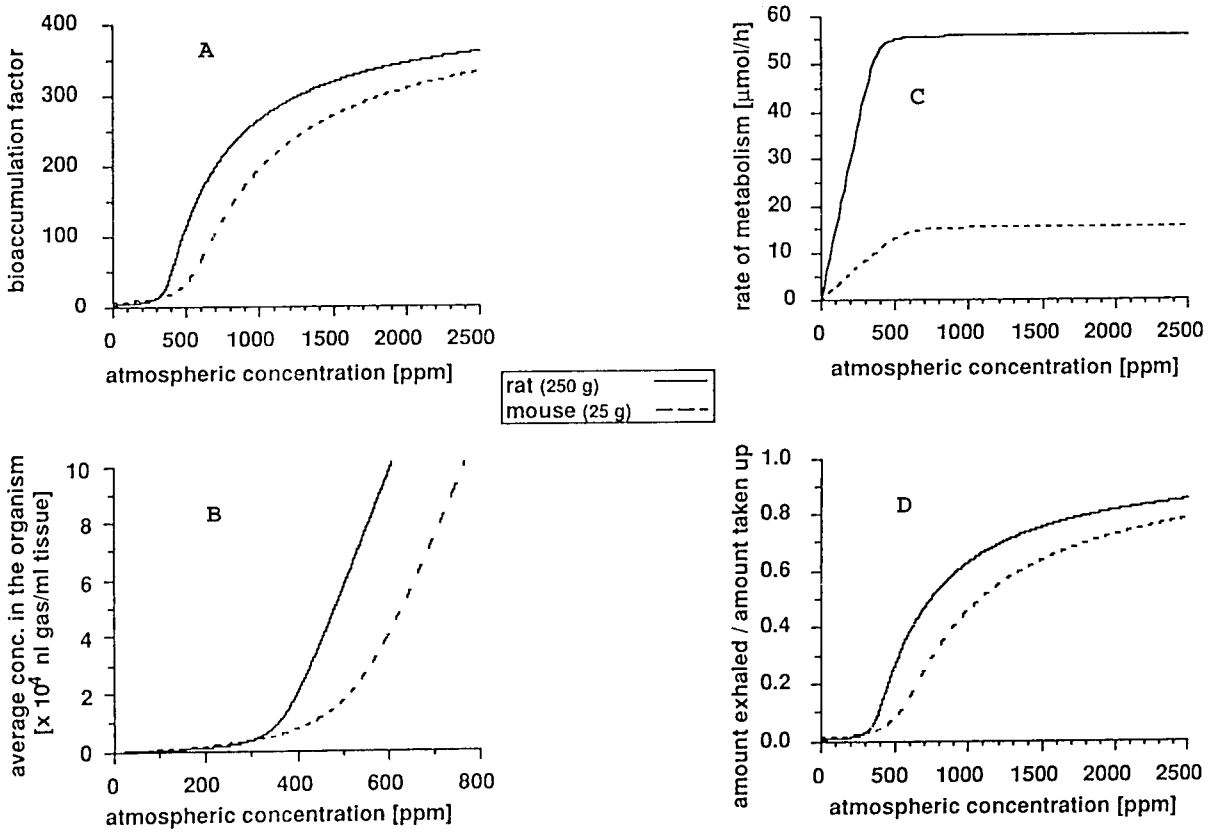


Fig. 4. Steady state toxicokinetic parameters of styrene calculated for one Sprague–Dawley rat (———) of 250 g and for one B6C3F1 mouse (- - - -) of 25 g [17]. A, bioaccumulation factor (K_{st}*) vs. atmospheric concentration of styrene (y_1); B, average concentration of styrene in the organism (y_2) vs. atmospheric concentration of styrene (y_1); C, rate of styrene metabolism (dNel/dt) vs. atmospheric concentration of styrene (y_1); D, ratio of amount of styrene exhaled to amount of styrene taken up vs. atmospheric concentration of styrene (y_1).

situation resulted in predicted values which were verified by comparison with direct determinations [17] with the exception of V_{max}, which was later confirmed by a human study carried out by Löf and Johanson [18]. For further discussion of results on the kinetics of styrene obtained by using the two-compartment model, see Ref. [17].

Since the two-compartment model possesses only a limited number of variables, these can be computed with high confidence. We were able to differentiate between several kinetic processes involved in the first metabolic steps of 2-nitropropane, n-hexane and n-heptane by analyzing gas uptake data for these compounds, measured in closed exposure chambers occupied by rats. For 2-nitropropane, a saturable pathway could be distinguished from a non-saturable one [19], the latter being strongly correlated with the solvent's hepatocarcinogenic activity, which was quantitatively investigated by means of the 'rat liver foci bioassay' [20,21]. For both alkanes n-hexane and n-heptane two different saturable metabolic processes were detected, one being characterized by high affinity and low capacity, one by low affinity and high capacity. Urinary excretion of 2,5-hexanedione, the neurotoxic metabolite of n-hexane correlated with the first process whereas the corresponding excretion of 2,5-heptanedione as a metabolite of n-heptane correlated with the second process. Metabolism to 2,5-hexanedione requires oxidation at the C2 positions only, which is preferred at low alkane concentrations; the biotransformation of n-heptane to 2,5-heptanedione needs oxidation at C3. This reaction is quantitatively less favoured [22].

The two-compartment model can be extended to the description of the body burden of metabolites resulting from exposure to the parent compound. This is exemplified in Fig. 5 on ethylene and its metabolite ethylene oxide, a mutagenic and carcinogenic epoxide. The two-compartment model for ethylene is linked to a two-compartment model for ethylene oxide, also a gaseous compound, which is eliminated by exhalation and by metabolism. The formation of ethylene oxide as the intermediate metabolite of ethylene was set to be equal to the product of the metabolic

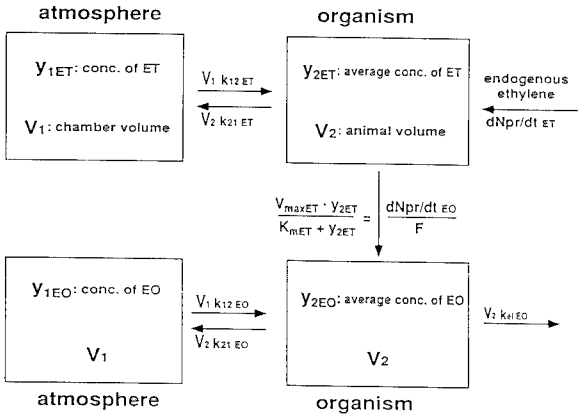


Fig. 5. Two-compartment model with physiological gas uptake for ethylene and ethylene oxide including the endogenous production of ethylene and its oxidative metabolism to ethylene oxide.

elimination rate of ethylene and of an extraction factor F ($F \leq 1$). The model was used to calculate the body burden of ethylene oxide in rats resulting from various conditions of exposure to ethylene or ethylene oxide gases [23]. For humans, the steady state level of hydroxyethylated N-terminal valine of hemoglobin resulting from endogenously produced ethylene was calculated and verified by direct measurements [24].

Furthermore, the two-compartment model proved to be excellent for studying the toxicokinetics of inhaled chemical mixtures. Since exposure to mixtures of styrene and 1,3-butadiene can occur especially in the manufacture of copolymers such as styrene-butadiene rubber, we have investigated the toxicokinetic interactions of both chemicals in rats co-exposed to atmospheric mixtures. In order to obtain values for V_{max}, Km_{app} and the inhibition constant, the measured data were analyzed using the two-compartment model [25] and, alternatively, a physiologically-based toxicokinetic model [26]. The results obtained by both models and the conclusion drawn are very similar: styrene inhibits the metabolism of 1,3-butadiene but 1,3-butadiene has no effect on the metabolism of

styrene. Therefore, co-exposure to both substances might result in a lower carcinogenic risk than exposure to 1,3-butadiene alone.

4. Physiologically-based toxicokinetic models

Physiologically-based models describe the organism by a set of compartments representing individual or lumped tissues and organs. The compartments are linked with each other by a circulating flow corresponding to the bloodstream represented by arterial and venous blood (Fig. 6). Following absorption of a chemical, it enters each compartment via the arterial blood and leaves it via the venous blood. The compartments are characterized by physiological parameters (as tissue volumes and blood flows through the compartments) and by physicochemical parameters (as the partition coefficients tissue/blood). For metabolizing organs, metabolic parameters (as V_{max} and K_m) are included. Generally, reference values for the physiological parameters can be taken from the literature. The partition coefficients of volatile compounds can be determined in vitro as mentioned above. The values for the metabolic parameters can be derived by fitting model simulated curves through concentration-time courses obtained in vivo (e.g. from blood, expired air and urine) or from in vitro experiments using fractions of organs or tissues (e.g. microsomes and cytosol).

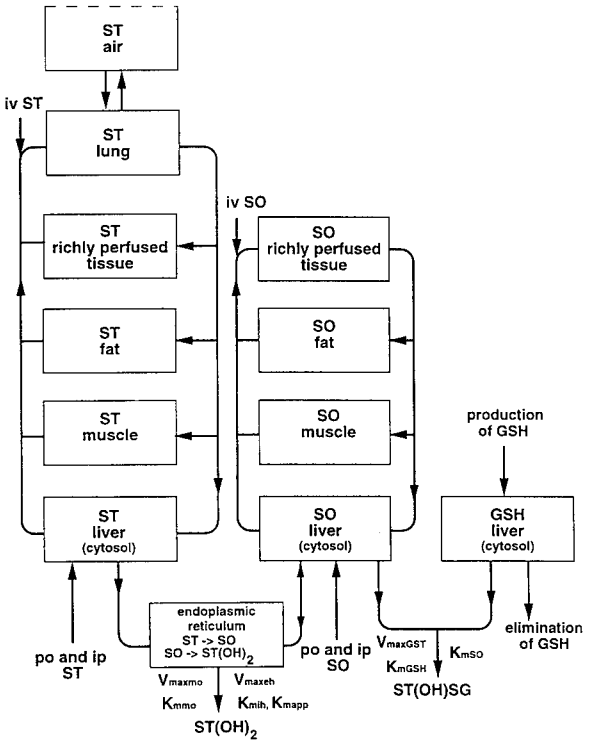


Fig. 6. Schematic structure of the physiologically-based toxicokinetic model used to describe the toxicokinetics of styrene and styrene-7,8-oxide in rodents and humans [7].

Physiologically-based toxicokinetic models are especially suitable for studying the influence of physicochemical, physiological and biochemical parameters on the toxicokinetics of a substance. Changes in the toxicokinetic behaviour become predictable by altering the appropriate parameters. Since the physicochemical and biochemical parameters can be obtained from in vitro determinations, physiologically-based toxicokinetic models are most helpful for predictions of species specific kinetics and also for understanding and interpreting observations.

As an example, Fig. 6 presents a physiologically-based toxicokinetic model which describes the toxicokinetics of styrene together with its reactive metabolite styrene-7,8-oxide (SO) in mice, rats and humans [7]. It is based on a model constructed for 1,3-butadiene and its metabolite butadiene monoxide [16]. The model includes inhalative uptake of styrene, its oxidation to SO in the hepatocellular endoplasmic reticulum, the immediate hydrolysis of a fraction of SO via endoplasmic epoxide hydrolase and the cytosolic conjugation of SO with glutathione.

This model allows the prediction of the fate of styrene and SO in blood, organs and tissues of mouse, rat and man resulting from various scenarios of exposure to styrene or SO. The curves plotted in Fig. 7 represent simulated SO blood concentrations in these species resulting from steady state exposures to atmospheric styrene concentrations of up to about 80 ppm (humans) and up to 800 ppm (rodents). The

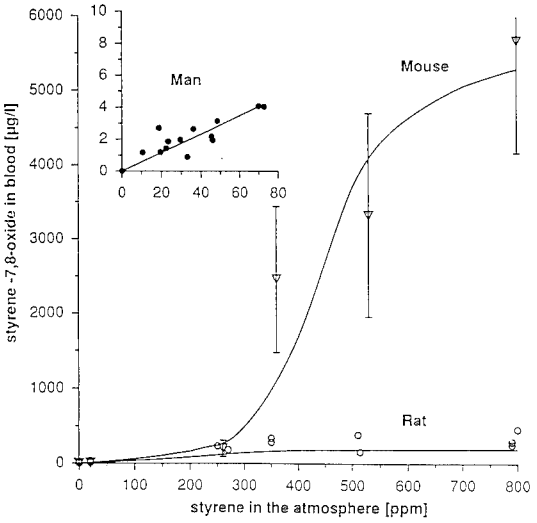


Fig. 7. Steady state concentrations of styrene oxide in blood of mouse, rat and man at exposure to constant concentrations of styrene vapors. Values for man [27], for rodents [28]; curves predicted by means of the model presented in [7].

curves can be compared to SO blood concentrations measured at steady state, also shown in the figure. At low concentrations, the ratio of the atmospheric styrene concentrations to the resulting SO blood concentrations is constant in the three species. In humans, no data resulting from styrene concentrations higher than 80 ppm are available. In rats, SO blood concentrations approach a maximum value at an exposure concentration of about 400 ppm styrene. It cannot be surpassed since styrene metabolism leading to SO becomes saturated. In mice however, a dramatic increase of SO in blood is predicted and also observed. It is the result of a complete depletion of the hepatocellular cytosolic glutathione in this species, originating from continuing biotransformation of styrene to SO even at high styrene concentrations at which styrene metabolism reaches V_{max}. Under these conditions, SO is detoxified by epoxide hydrolase activity almost exclusively. Even this enzyme reaches the limits of its capacity being unable to compensate for the loss of the glutathione dependent pathway. Therefore, small interanimal differences in V_{max} of hepatocellular endoplasmic epoxide hydrolase result in significant differences in the individual SO blood concentrations in mice leading to high inter-animal variances. All these effects have been predicted by the model and have been verified by the data shown in Fig. 7 and by a series of further in vivo measurements [7].

5. Disadvantages and advantages of both model structures

Since the two-compartment model describes the organism by a single homogenous compartment, it has an inherent structural limitation: Multicompartmental phenomena like redistribution from a storage compartment (e.g. redistribution of styrene from fatty tissue into blood) or simultaneous metabolism in different tissues cannot be distinguished by this model. Its advantage over a physiologically-based toxicokinetic model is the reduced uncertainty (higher safety) of the parameters obtained by curve fits, which results from the limited number of variables and also the independent estimation of inhalation and exhalation clearances due to the closed chamber technique. The model allows the quantification of processes representing invasion, accumulation, excretion and biotransformation. Additionally, it can be used to calculate tissue concentrations at steady state if the partition coefficients tissue/air are known.

In contrast, use of physiologically-based toxicokinetic models for estimating numerical values of model parameters by fitting simulated curves through measured data is unsatisfactory, since the values obtained are highly influenced by the structure of the model and by inaccuracies of the physiological and physicochemical parameters included in the model. However, physiological models are more imaginable, since they allow the incorporation of physiologically and biochemically relevant mechanisms in the model structure resulting in deeper insights into the toxicokinetic processes. In particular, concentrations in different tissues or organs due to rapidly changing exposure conditions (e.g. daily or weekly variations of exposure at work place) can be simulated by such models. Perhaps the most important application could lie in their usability for predictions of species specific kinetics from in

vitro data and for helping to explore and understand the mechanisms leading to the toxic action.

6. Conclusion

Different approaches can be used for the study of the toxicokinetics of gaseous compounds. The choice of the appropriate model should be based on the problems to be solved.

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PBK modeling for metals. Examples with lead, uranium, and chromium

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Abstract

Physiologically-based models for metals differ in several key respects from models for organic compounds. Although sequestration by binding to specific metal-binding proteins in liver, kidney, and red cell may be important, neither the magnitude and pattern of metabolism nor potential accumulation in fat is a component of models of metal kinetics. In addition, the long residence times of bone-seeking elements require that bone turnover and metabolism be incorporated into physiologically-based models for these elements. Three mechanisms (rapid exchange at bone/blood interfaces, trapping or incorporation with forming bone and loss with resorbing bone, and slow exchange throughout the total bone volume) are potentially important in the overall interchange of bone-seeking elements between blood and bone. Three examples are given of applications of physiologically-based kinetic models for the bone-seeking elements lead, chromium, and uranium to assist in answering practical questions relating to bioavailability, distribution, and data interpretation.

Keywords: PBK models, metals; Lead; Uranium; Chromium

1. Introduction

The components of physiologically-based models of the kinetic behavior of metals are quite different from the components of physiologically-based models of the behavior of drugs or solvents, for which the nature and magnitude of metabolism are integral to the model and uptake into fat is frequently a driving force in disposition. Although they may change oxidation state in the body or shift in their association with organic ligands, metals are not metabolized in the sense in which organic chemicals are. They do not accumulate preferentially in fat but they may accumulate in other tissues, most commonly the liver or the kidney, sometimes in association with specific metal-binding proteins. They may be sequestered in the red cell in such a way that plasma becomes the driving force for their entry into tissues. In addition, certain elements are incorporated into bone, either by trapping in some fashion (aluminum, chromium) or by substitution for constituents of the bone crystal matrix (uranium, strontium, lead). These elements have long residence times in the bone and consequently in the body. Therefore, especially if ambient exposure is significant, it is necessary to include anatomic and physiologic changes associated with growth, and in particular of bone growth, in any physiologically-based model for

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bone-seeking elements. The three examples of applications of physiologically-based models given in this paper, for the bone-seeking elements lead, chromium, and uranium, are based on a physiologic model of rat bone growth and turnover [1].

Bone-seeking elements are taken up into and released from bone by three principal mechanisms. One is rapid exchange at all surfaces of the bone in contact with blood vessels, one is incorporation into forming bone and return to the blood with resorbing bone, and the third, which applies only to those elements whose size and charge density allow them to substitute for constituents of the crystal matrix, is a heteroionic exchange process that can be modeled as diffusion throughout the entire bone volume. The quantitative importance of this slow exchange mandates consideration of the timing and pattern of exposure, and therefore also of growth and aging. These three mechanisms for the exchange of metals in bone are included as required in the physiologically-based models used in the applications below, along with interchanges in liver, kidney, lung, richly-perfused tissues, and poorly-perfused tissues, and excretion from liver and/or kidney. All processes are linked to lean body weight and/or to its rate of change.

2. Application of model in bioavailability studies

The purpose of these studies was to estimate the fractional bioavailability of lead from mine waste-containing soils through an exposure in the gastrointestinal tract. The experimental design [2] was a conventional one. Groups of male and female Sprague–Dawley rats, 7–8 weeks old at the start of the study, were administered one of eight different dose levels of the mine waste soil, admixed in their diet, for 30 days. The 30-day exposure period was chosen because blood lead concentration reaches an approximate apparent steady state after that time, although it has not in fact reached steady state relative to bone lead. Other groups were given one of five different dose levels of the soluble salt lead acetate, while the reference dose group was administered lead acetate intravenously, daily for 30 days. At the end of the dosing period, blood, liver, and bone (femur) lead were assayed in all rats.

In the standard method for estimation of bioavailability, tissue concentrations in the mine waste-fed animals are compared with tissue concentrations in the lead acetate-fed animals; these concentrations, in turn, are referenced to tissue concentrations in the intravenous lead acetate groups. The results are shown in Table 1. The three highest mine waste dose groups could not be used in this calculation because tissue concentrations exceeded those observed in the lead acetate groups. As illustrated in Table 1, estimated bioavailability varies with the tissue. There is no systematic dependence of estimated bioavailability on dose.

These data were reanalyzed using a physiologically-based model of lead kinetics [3]. The model was fit simultaneously to blood and bone lead concentrations after the 30-day oral exposure, allowing only fractional absorption from the gastrointestinal tract to vary. The single fractional absorption value that gave the best fit to blood and bone lead concentrations at each dose was

Table 1
Absolute percent bioavailability of lead from mine waste-containing test soils added to feed of juvenile male Sprague–Dawley rats. Conventional calculation

Lead dose, ppm	Lead dose, mg/kg/day for 30 days	Bioavailability determined from		
		Blood concentrations	Bone concentrations	Liver concentrations
1.62	0.124	1.3 (9.0)	0.36 (1.6)	0.26 (1.3)
4.05	0.314	2.3 (3.1)	0.48 (0.64)	1.4 (0.75)
7.82	0.680	1.3 (1.4)	0.09 (0.34)	0.12 (0.32)
16.2	1.20	2.2 (0.8)	0.48 (0.25)	0.45 (0.25)
19.5	1.67	1.6 (0.6)	0.35 (0.16)	0.11 (0.15)

Standard Error in parentheses.

Table 2
Absolute percent bioavailability of lead from mine waste-containing test soils added to feed of juvenile male Sprague–Dawley rats. Estimated using PBK model

Lead dose, ppm	Lead dose, mg/kg/day for 30 days		Bioavailability	
	Males	Females	Males	Females
7.82	0.680	0.916	1.60	–
16.20	1.20	1.88	1.20	0.90
19.50	1.67	2.31	0.95	0.85
40.50	3.36	4.40	0.57	0.41
78.20	5.42	7.63	0.52	0.37
195.0	12.7	23.8	0.30	0.24

designated the fractional bioavailability at that dose. Growth of the juvenile animals was taken into account in simulating the 30-day exposure. The results of the reanalysis are given in Table 2. The two lowest dose groups of males, and the three lowest of females, were excluded from the reanalysis because tissue concentrations were essentially indistinguishable from concentrations in the control group (background concentrations). A systematic dose dependence of fractional absorption emerges from the remainder of the data set, with fractional absorption decreasing as the dose increases.

While it appears from the results as presented in Table 2 that fractional absorption was lower in the females than in the males, this is not the case. The female rats in this study were eating more than the males. When dose rate is expressed as the amount of lead ingested per unit body weight per day for male and female rats (Fig. 1), both the smooth progression from higher to lower fractional absorption with increasing dose and the absence of any gender dependence are apparent. The advantages of using the physiologically-based model for the purpose of estimating fractional bioavailability, then, are that the model integrates tissue concentrations from each dose group into a single estimate of bioavailability, that it gives absolute bioavailability directly without need for either intravenous or oral reference exposure groups, and that it does not require dosing to steady state.

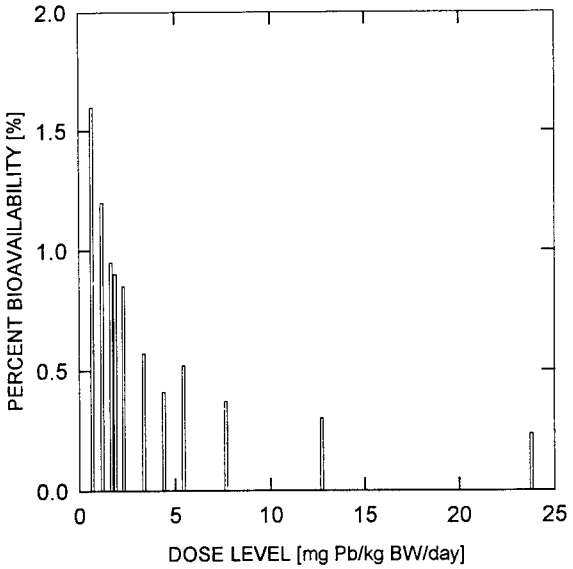


Fig. 1. Dependence of fractional bioavailability of lead from mine waste soil on rate of lead ingestion in male and female rats. Reproduced with permission from Polák et al. [3].

3. Application of model to assist with interpretation of distribution data

The oxidation state of chromium raises risk assessment issues. The carcinogenicity, and possibly the toxicity, of chromium appear to be due to generation of reactive intermediates in the process of reduction of Cr(VI) to Cr(III). Reduction is rapid in both the lung and the gastrointestinal tract. Consequently, whether significant amounts of Cr(VI) are able to reach tissues unchanged is an important question.

A number of published data sets were used in the development and calibration of a physiologically-based model of chromium kinetics [4]. One of the most complete is that of Weber [5]. In this study, adult male Sprague–Dawley rats were

given a soluble ^{51}Cr salt, sodium dichromate, as a single intratracheal administration. Multiple tissues were monitored for their total chromium concentration for 40 days following the exposure. Fig. 2 shows the profile of liver total chromium content along with the corresponding simulation fit to these data. Given the relative rates of Cr(VI) and Cr(III) transfer into tissues, the liver data can be fit only if Cr(VI) is allowed to account for the initial peak. Alternative explanations – for example, absorption of Cr(VI) transferred from the lung to the gastrointestinal tract – were examined and found to be incapable of generating the observed initial peak. The simulation, broken down into its Cr(VI) and Cr(III) components, is illustrated in Fig. 3. The advantage of using a physiologically-based model to assist with interpretation of these data is that its basis in physiology and in the known membrane transfer properties of Cr(III) and Cr(VI) allows the kinetic behavior of total chromium to be factored into the separate contributions of the two oxidation states.

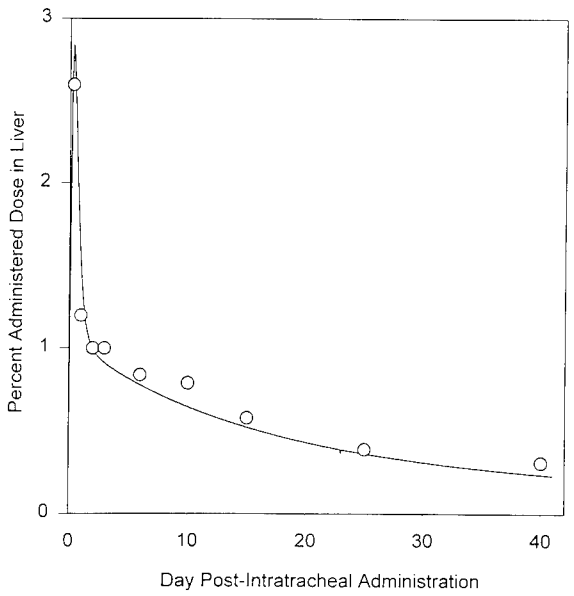


Fig. 2. Amount, as percent of administered dose, of total chromium in liver of rats given a single intratracheal dose of sodium dichromate. Data from Weber [5]. The line is the simulation. Reproduced with permission from O'Flaherty [4].

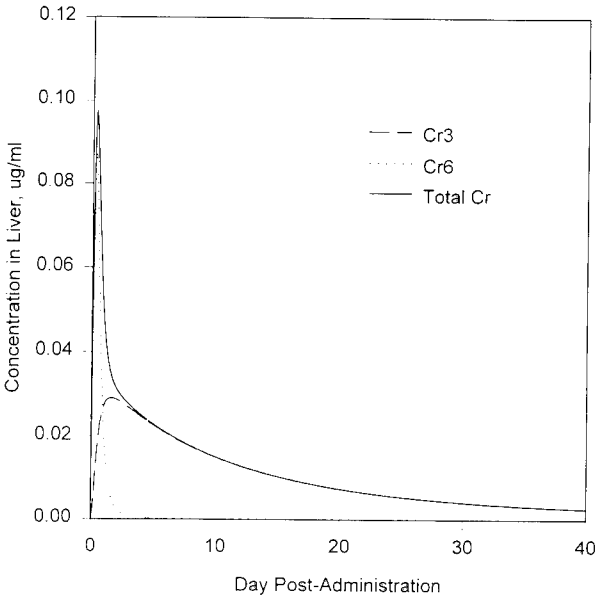


Fig. 3. The simulation of Fig. 2 (solid line), broken down into its Cr(VI) (dotted line) and Cr(III) (dashed line) components.

4. Application of model to explain anomalous data

Uranium, like lead, is a bone volume-seeking element. As part of a larger study whose purpose was to determine whether bone uptake of uranium might be a factor in its chronic renal toxicity [6], uranium was administered to rats for 14 days by means of an osmotic pump implanted intraperitoneally. Key tissues were monitored for their uranium content before, during, and for 100 days after the 14-day exposure period. A variant of the physiologically-based lead model was calibrated to these concentration data. In order to estimate urinary clearance for model calibration, rats were placed in metabolism cages overnight with free access to water but not to food. The relationship of the rate of urinary uranium excretion to the concentration of uranium in the blood is illustrated in Fig. 4. Urinary uranium clearance was estimated to be 0.7 l/day.

When the values of other model parameters were optimized by fitting the model simultan-

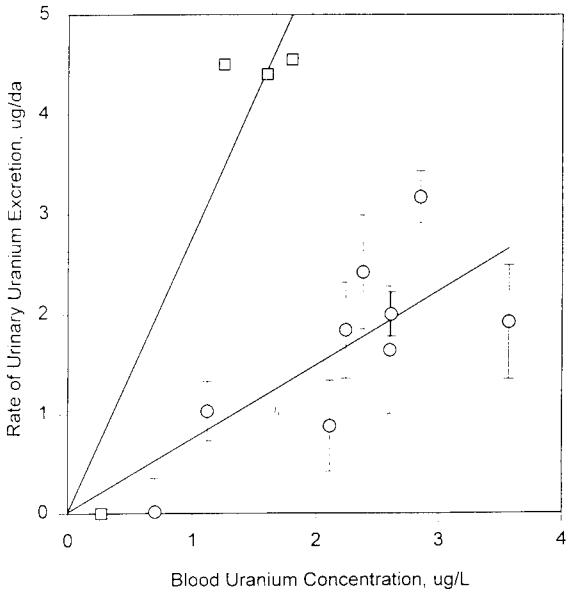


Fig. 4. Relationship of the rate of urinary uranium excretion to the concentration of uranium in the blood. ○, the means and standard deviations of observations in three rats, are from a study design in which the rats were fasted overnight during urine collection; □, data from three uranium-exposed and two control rats allowed free access to food during urine collection.

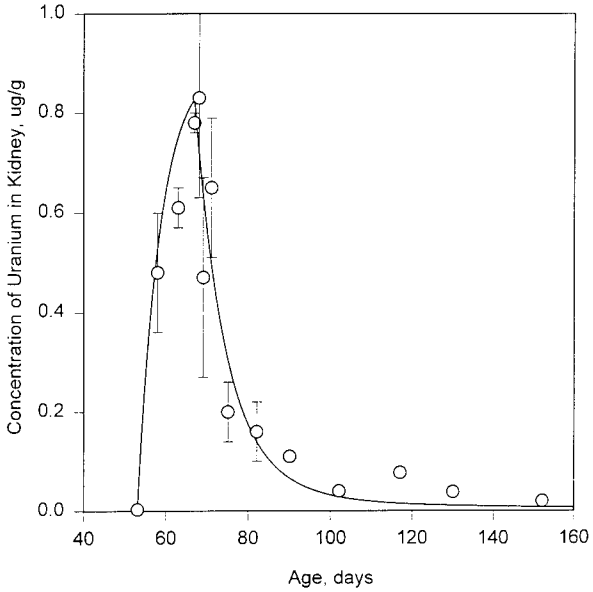


Fig. 5. Optimized model fit to kidney uranium concentrations during and after 14-day exposure of rats by means of an osmotic pump implanted intraperitoneally. Data are the means and standard deviations of observations in three rats.

eously to tissue and urine concentrations, the model was found to be incompatible with the concentration data. Fig. 5 illustrates the optimized model fit to kidney concentrations. While tissue concentrations could be fit well independently of urine concentrations, simulated urinary excretion had to be much higher than observed excretion in order to achieve these fits (Fig. 6). The nature of the model incompatibility, then, suggested that the previously-estimated value of urinary clearance might be incorrect.

The urinary clearance experiment was repeated, with three rats housed singly in metabolism cages for the duration of the 14-day osmotic pump exposure period. During this time, the rats had free access to water and to a controlled amount of food, 18 g/day, adequate to maintain growth but low enough to discourage scattering of feed about the cages. Urinary clearance was estimated by means of 24-h urine collections on the last day of exposure. Fig. 4 shows the rate of

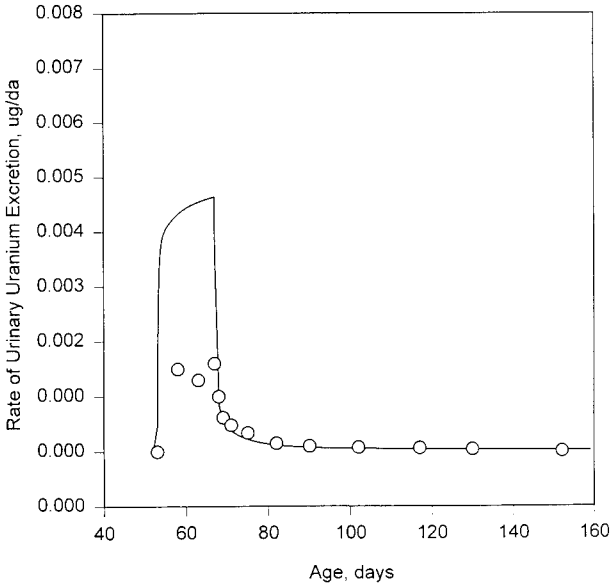


Fig. 6. Simulated rate of urinary uranium excretion required in order to achieve good tissue concentration fits as exemplified by Fig. 5, compared with observed urinary excretion during urine collection from rats fasted overnight. Data are the means of observations in three rats.

urinary uranium excretion in these three rats. The corresponding uranium clearance was calculated to be about 3 l/day, or more than four times the value previously estimated. When the time course of urine uranium concentration is simulated by alternating the larger clearance value during periods of free access to food with the lower clearance value during housing in metabolism cages without access to food (Fig. 7), the adjusted simulation reproduces well the urine concentration profile originally observed. In this study, therefore, application of the physiologically-based model correctly suggested the source of a fault in the original experimental design.

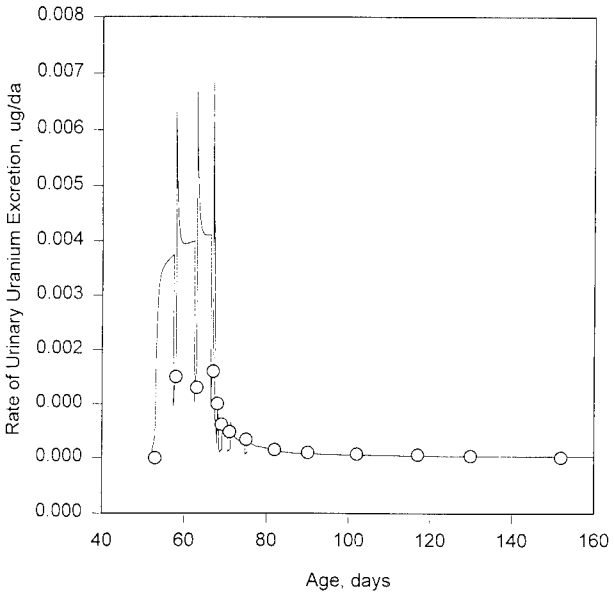


Fig. 7. Rate of urinary uranium excretion during and after 14-day exposure of rats by means of an osmotic pump implanted intraperitoneally. Excretion was simulated by alternating periods of higher (3.0 l/day) urinary clearance while the rats had access to food with periods of lower (0.7 l/day) urinary clearance during overnight fasting. Data are the means of observations in three rats.

These three examples of practical applications of physiologically-based kinetic models for elements incorporated into bone illustrate how such models can be used to assist in interpreting experimental data and in suggesting sources of model incompatibility with data.

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Case studies of the use of biomarkers to assess exposures

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Abstract

Because many environmental toxicants are ubiquitous, humans are continuously exposed to them. At other times, certain populations may be more highly exposed to these toxicants from point sources. The evaluation of the degree of the exposure to either a population or an individual is frequently based on indirect surrogates of exposure, such as questionnaire data on time-activities and/or concentrations measured in environmental media. We prefer to assess the degree of the exposure to a given toxicant by measuring the concentration of the toxicant, its metabolite(s), or reaction product(s) in human specimens. Then by applying pharmacokinetic information for that toxicant, we can best reconstruct the exposure scenario. These data are then compared to reference range levels of these toxicants in the preferred biologic specimen. The development and uses of the reference range data are exemplified by case studies including potential exposure to dioxin and solvents.

Keywords: Internal dose; Reference range levels; VOCs; Dioxin

1. Introduction

Reference ranges for biomarkers are used extensively in clinical medicine, e.g., cholesterol, triglyceride, and various enzyme levels. Safety levels of urinary and blood biomarkers of exposure are used to a lesser extent to help ensure safety in the workplace, e.g., the Biological Exposure Indices (BEI) in the United States and the MAK values in Germany. However, reference levels of exposure biomarkers have not been used to the same extent in environmental health. We have developed reference range levels for 32 volatile organic compounds (VOCs) in human blood and 12 urinary pesticides or their metabolites in the general population of the United States as part of our priority toxicant reference range study [1]. In other epidemiologic studies, we have compared levels of the internal dose of environmental toxicants or their metabolites in potentially exposed populations with other referent populations such as controls. Establishment and applications of these reference data are described in several epidemiologic studies that involve different classes of environmental toxicants.

2. Volatile organic compounds (VOCs)

Many volatile organic compounds (VOCs) are ubiquitous in the environment. They have been

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shown to exist in higher concentrations in indoor air than in outdoor air [2]. Reported health effects from exposure to VOCs have included eye irritation, sick-building syndrome, neurologic effects, and cancer. Centers for Disease Control and Prevention (CDC) scientists developed an isotope-dilution purge and trap gas chromatography/mass spectrometry method to quantify 32 VOCs (Table 1) in 10 ml of blood with detection limits in the parts-per-trillion range [3]. This is a

Table 1
Biological monitoring measurements currently performed at the National Center for Environmental Health (NCEH) of the Centers for Disease Control and Prevention (CDC)

Metals[a]	
Lead	Beryllium
Mercury	Chromium
Cadmium	Nickel
Arsenic	Thallium
Vanadium	
Polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, coplanar polychlorinated biphenyls (PCBs)[b]	
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	1,2,3,4,7,8-Hexachlorodibenzofuran (H_xCDF)
1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (P_nCDD)	1,2,3,6,7,8-Hexachlorodibenzofuran (H_xCDF)
1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin (H_xCDD)	1,2,3,7,8,9-Hexachlorodibenzofuran (H_xCDF)
1,2,3,6,7,8-Hexachlorodibenzo-*p*-dioxin (H_xCDD)	2,3,4,6,7,8-Hexachlorodibenzofuran (H_xCDF)
1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin (H_xCDD)	1,2,3,4,6,7,8-Heptachlorodibenzofuran (H_pCDF)
1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin (H_pCDD)	1,2,3,4,7,8,9-Heptachlorodibenzofuran (H_pCDF)
1,2,3,4,6,7,9-Heptachlorodibenzo-*p*-dioxin (H_pCDD)	1,2,3,4,6,7,8,9-Octachlorodibenzofuran (OCDF)
1,2,3,4,6,7,8,9-Octachlorodibenzo-*p*-dioxin (OCDD)	3,3′,4,4′-Tetrachlorobiphenyl (TCB)
2,3,7,8-Tetrachlorodibenzofuran (TCDF)	3,4,4′,5-Tetrachlorobiphenyl (TCB)
1,2,3,7,8-Pentachlorodibenzofuran (P_nCDF)	3,3′,4,4′,5-Pentachlorobiphenyl (P_nCB)
2,3,4,7,8-Pentachlorodibenzofuran (P_nCDF)	3,3′,4,4′,5,5′-Hexachlorobiphenyl (H_xCB)
Volatile organic compounds (VOCs)[c]	
1,1,1-Trichloroethane	Chlorobenzene
1,1,2,2-Tetrachloroethane	Chloroform
1,1,2-Trichloroethane	*cis*-1,2-Dichloroethene
1,1-Dichloroethane	Dibromochloromethane
1,1-Dichloroethene	Dibromomethane
1,2-Dichlorobenzene	Ethylbenzene
1,2-Dichloroethane	Hexachloroethane
1,2-Dichloropropane	*m*-/*p*-Xylene
1,3-Dichlorobenzene	Methylene chloride
1,4-Dichlorobenzene	*o*-Xylene
2-Butanone	Styrene
Acetone	Tetrachloroethene
Benzene	Toluene
Bromodichloromethane	*trans*-1,2-Dichloroethene
Bromoform	Trichloroethene
Carbon Tetrachloride	
Chlorinated pesticides and non-coplanar polychlorinated biphenyls[d]	
Aldrin	DDE
Chlordane, alpha	DDT
Chlordane, gamma	Dieldrin
beta-Hexachlorocyclohexane	Endrin
gamma-Hexachlorocyclohexane	Heptachlor
Biphenyls, polychlorinated (total)	Heptachlor epoxide
Biphenyls, polychlorinated (individual congeners)	Hexachlorobenzene
DDD	Mirex
trans-Nonachlor	Oxychlordane

Table 1 (*continued*)

Non-persistent pesticides[c]	
Urine metabolites	*Parent pesticides*
2-Isopropoxyphenol (IPP)	Propoxur
2,5-Dichlorophenol (25DCP)	1,4-Dichlorobenzene
2,4-Dichlorophenol (24DCP)	1,3-Dichlorobenzene, dichlofenthion, prothiofos, phosdiphen
Carbofuranphenol	Carbofuran. benfuracarb, carbosulfan, furanthiocarb
2,4,6-Trichlorophenol (246TCP)	1,3,5-Trichlorobenzene, hexachlorobenzene, lindane
3,5,6-Trichloro-2-pyridinol (TCPY)	Chloropyrifos, chlorpyrifos-methyl
4-Nitrophenol (4NP)	Parathion, methyl parathion, nitrobenzene, EPN
2,4,5-Trichlorophenol (245TCP)	1,2,4-Trichlorobenzene, fenchlorphos, trichloronate
1-Naphthol (1NAP)	Naphthalene, carbaryl
2-Naphthol (2NAP)	Naphthalene
2,4-Dichlorophenoxyacetic acid (24D)	2,4-D
Pentachlorophenol (PCP)	Pentachlorophenol
Dicamba	Dicamba

[a] Urine or blood sample, 3 ml; typical limit of detection, low parts-per-billion.
[b] All analytes measured in serum from one 25-ml blood sample if exposure is near background levels; smaller samples are adequate for higher exposures; typical limit of detection, low parts-per-trillion on a lipid-weight basis, low parts-per-quadrillion on a whole-weight basis.
[c] All analytes measured in one 10-ml blood sample; typical limit of detection, low parts-per-trillion.
[d] All analytes measured in serum from one 5-ml blood sample; typical limits of detection, low parts-per-billion.
[e] All analytes measured in one 10-ml urine sample; typical limits of detection, low parts-per-billion.

full-scan method at 3000 resolving power, so that in addition to acquiring quantitative data on these 32 VOCs, many additional VOCs can be qualitatively identified and in many cases, quantified [4].

CDC, with financial support from the Agency for Toxic Substances and Disease Registry (ATSDR), selected a 1000-person subset of the NHANES III population to determine reference ranges for these 32 VOCs. The 1000 people were chosen from both genders, all regions of the contiguous U.S., urban/rural residents and were adults between 20 and 59 years of age [1]. The data showed that 11 of these VOCs were measured in more than 75% of the people. The non-chlorinated aromatics, including styrene, toluene, ethylbenzene, *o*-xylene, *m,p*-xylene, and benzene, which is a known human carcinogen, were the most prevalent. The primary sources of these compounds are tobacco smoke and exhaust from internal combustion engines. The xenobiotic found at the highest concentration and highest frequency was 1,4-dichlorobenzene [5]. The blood exposure data for this moth repellent and room deodorizer correlated highly with urinary levels of its primary metabolite, 2,5-dichlorophenol [6].

Five of the VOCs were found in 10%–75% of the selected population, whereas the remainder of the VOCs were either nondetectable or were detectable in less than 10% of the specimens tested. Thus, finding this latter group to any significant extent in case populations is important in human effect studies. These analytical methods and reference range studies have been applied to a wide variety of case studies and population studies. These include exposure assessment studies of toxic waste sites, oil-well fires [7], sick building syndrome [5], multiple chemical sensitivity, and oxygenated fuels involving methyl tertiary-butyl ether (MTBE) [8]. In each of these examples, the blood concentrations of VOCs were compared with the reference range population data.

However, pharmacokinetic data are needed to properly interpret blood levels of VOCs. Scientists from CDC and EPA have collaborated in determining the half-lives of many VOCs in humans subjected to low-level mixtures of VOCs in well-controlled chamber studies. The blood

half-lives were less than 0.5 h, but the elimination time courses were multiexponential, thereby suggesting multiple storage sites within the body. The blood uptake portion of the 4-h exposure curve exhibited a rapid uptake that reached a plateau after about 50 min; the uptake rate was not concentration-dependent, but the blood concentration was directly dependent on the air concentration. When exposure ceased after 4 h, the decay was rapid, but the decay rate also reached a plateau after about 1 h; however, the VOC levels remained elevated even 24 h after exposure as compared with the pre-exposure blood levels. Thus, like those compounds with long biologic half-lives, such as dioxin, VOCs also can be the focus of exposure assessment studies, if the blood samples are collected within 1 day following exposure.

3. Dioxin: Operation Ranch Hand study

From 1962 through 1970 during the Vietnam Conflict, the main mission of the U.S. Air Force's Operation Ranch Hand was to spray defoliants, such as Agent Orange, over densely vegetated areas of South Vietnam. Agent Orange consisted of an equal mixture of 2,4-D and 2,4,5-T in diesel oil; the 2,4,5-T was contaminated with 2,3,7,8-TCDD (dioxin) in the parts-per-million range. Dioxin is lipid soluble and thus tends to be stored in the lipid-rich depots of the human body. Dioxin has a long half-life – more than 7 years in humans [9,10]. In 1982, the Air Force began a prospective cohort study, specifically looking at health, reproductive, and mortality outcomes that might be associated with exposure to Agent Orange and other herbicides containing dioxin. These health studies will be performed on the veterans of Operation Ranch Hand every 5 years through the year 2002. One of the first tasks was to develop an exposure index in order to classify each veteran's exposure; this index would then be used as the basis for exposure and for correlating with any health effects.

This exposure scenario was similar to that of exposure in an occupational setting in that the primary exposure was thought to be direct exposure to the herbicide itself, rather than exposure through an environmental pathway. The exposure index consisted of the average concentration of dioxin in the Agent Orange during one's tour of duty multiplied by the number of gallons of Agent Orange sprayed during one's tour divided by the number of men in one's specialty during that period. The total number of eligible men in the study was limited to the 1200–1300 survivors of the Operation. The U.S. Air Force and various review boards believed that this exposure index not only could serve as a reliable basis for assessing exposure to dioxin but that any noted adverse health effects could be related to this index.

In 1987, the U.S. Air Force contracted with our laboratory to analyze 150 serum samples from Operation Ranch Hand veterans in order to determine if these veterans had been unduly exposed to dioxin and to compare the Air Force's exposure index with the measured internal dose of the veterans. We found that there was a wide range of dioxin levels and that several were orders of magnitude higher than control [11]. In addition, there was essentially no correlation between the exposure index and the serum dioxin level [12]. Because of this finding, the Air Force further contracted with CDC to analyze the serum of all surviving members of Operation Ranch Hand, and this serum dioxin level became the exposure index used to correlate with any adverse health effects [11]. Had the Air Force used its original exposure index for the Operation Ranch Hand study, a great deal of misclassification would have resulted, and any health effect conclusions of the study would have been invalid.

4. Dioxin: U.S. Army ground troops in Vietnam

The chemical of concern was again the dioxin in Agent Orange. The potential environmental pathways were skin contact with and inhalation of the spray containing the herbicide, skin contact with sprayed vegetation and soil, and ingestion of water and food that had been sprayed. The amount of dioxin in the Agent Orange from

1966–1969 was known. The duration of contact was gathered from questionnaires given to the veterans and from U.S. military records containing the locations of military units, the locations where herbicide was sprayed, and the dates when the herbicide was sprayed.

Six exposure indices were generated from this information; four of the indices were based on a soldier's potential for exposure from direct spray or on his being located in an area that had been sprayed within the previous 6 days; the other two exposure indices used self-reported data and included an index that was based on the veteran's perception of to how much herbicide he had been exposed. To test the validity of these exposure indices, CDC measured blood-dioxin levels in 646 enlisted ground troop veterans who had served in III Corps for an average of 300 days during 1966–1969. For comparison, blood dioxin levels, in 97 non-Vietnam U.S. Army veterans who served during the same time were also measured [12].

The results showed no meaningful association between dioxin levels and any of the exposure indices. The mean, median, and frequency distributions for both the Vietnam and non-Vietnam veterans were remarkably similar; the study had a 95% statistical power to detect a difference of only 0.6 ppt in the medians, but this difference was not found. This finding exemplifies the value of measurements of internal dose in exposure assessment. It also points out the need to develop specific and sensitive methods, for if the detection limit for dioxin had been 20 ppt (lipid adjusted), then almost all the results would have been nondetectable. Furthermore, because elevated exposures could not be documented, plans for a prospective cohort health study were dropped.

5. Dioxin: occupational setting

CDC's National Institute of Occupational Safety and Health (NIOSH) conducted a retrospective study to evaluate health outcomes, including mortality from cancer, among more than 5000 workers who may have been occupationally exposed to dioxin, as a result, for example, of the production of 2,4,5-trichlorophenol [14]. Many of these workers were deceased. Because many were deceased and because of the large number of potentially exposed men, NIOSH epidemiologists had to develop an exposure index for use in correlating exposure with the health outcomes (the effect). Serum dioxin measurements were performed on 253 workers; the results, which showed serum dioxin levels from nondetectable to greater than 300 ppt, were compared to various exposure indices. From this analysis, epidemiologists determined that the best exposure index was years of work in a job with potential exposure. Since this exposure index had been validated to and calibrated with serum dioxin levels, it could be used as the exposure index in this study and exposure and effects could be compared directly with those found in other studies. This process again demonstrates the need for measuring the internal dose in exposure assessment or health effect studies and for comparing the serum dioxin levels in potentially exposed levels to reference levels.

6. Summary

We have demonstrated the development and utilization of internal dose levels of environmental toxicants. We have developed reference range data for a variety of other environmental toxicants (Table 1). We believe that such data have a variety of practical uses, such as in risk assessments and also for prioritizing chemicals to be studied in programs such as the National Toxicology Program and the Toxic Substances Control Act.

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Strategies for use of biological markers of exposure

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Abstract

A major public health concern is the degree to which environmental or occupational exposures to exogenous chemicals result in adverse health effects. Biological markers have the potential for helping to answer this important question by providing links between markers of exposures and markers of early stages of the development of disease. However, that potential requires in-depth, mechanistic research to be fully realized. Biological markers of exposure have been extensively investigated, and mathematical models of the toxicokinetics of agents have been developed to relate exposures to internal doses. The field of clinical medicine has long used clinical signs and symptoms to detect disease. However, the critical area of research needed to improve the application of biomarkers to environmental health research is mechanistic research to link dose to critical tissues to the development of early, pre-clinical signs of developing disease. Only if the mechanism of disease induction is known can one determine the 'biologically effective' dose and the earliest biological changes leading to disease.

Keywords: Biological markers; Toxicokinetics; Pharmacodynamics; Modeling; Markers of exposure; Markers of effects

1. Biological markers of exposure

Biological markers of exposure are exogenous substances or their metabolites, or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism [1]. Because biomarkers of exposure are all measures of internal substances, they are biological markers of dosimetry that are the result of exposures (Fig. 1). As shown in Fig. 1, one could potentially have biomarkers for each of the indicated steps that link an exposure to a clinical disease. Even the markers of steps that are not directly in the line leading to the disease process can be useful, if one can link them quantitatively to steps leading to disease. For example, if one knows the quantitative relationship between levels of hemoglobin adducts for a specific chemical (example of noneffective macromolecular adducts) and biologically effective liver DNA adducts (example of a biologically effective dose) for a liver carcinogen, one could theoretically use the more available blood adducts as a predictor of the biologically effective dose.

The emphasis on quantitation is to meet the requirements for setting regulations for allowable exposures and for predicting the likelihood of adverse health effects. If one only needs to know if an exposure has occurred, the presence of a biological marker specific for the chemical of concern may be all that is needed. However, for

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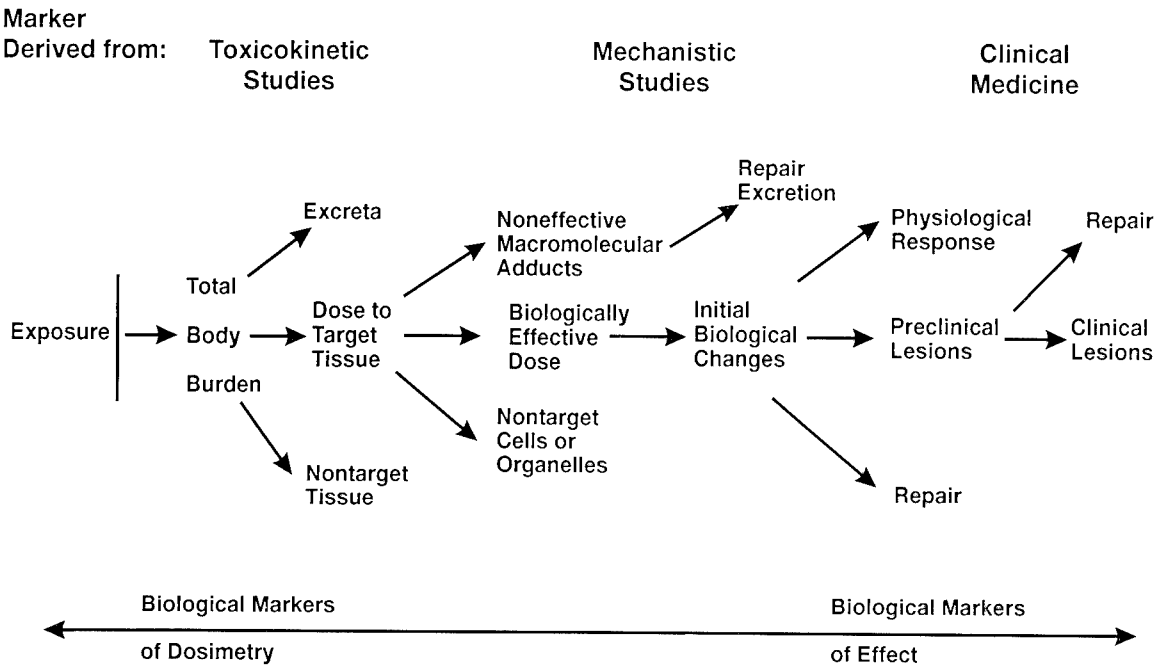


Fig. 1. Biomarkers for risk assessment. Toward the left are biomarkers of dosimetry resulting from exposures; most of these markers represent values obtained from toxicokinetic studies. Toward the right are biological markers of effect; many of these markers are standard signs and symptoms familiar to clinicians. One of the greatest needs in biomarker research is to obtain more information on the link between biologically effective doses and the early, initial biological changes that can lead to disease; such values will come from studies on mechanism of disease induction.

the purposes of risk assessment, that is, determining the potential for a given exposure to an exogenous substance to cause adverse health effects, one needs a biomarker that can be (1) quantitatively related to prior exposures to a specific chemical and (2) can be quantitatively related to, or predictive of, later developing disease. Strategies to meet these needs are described below.

2. Strategies for use of biological markers of exposure to assess prior exposures

Many commonly measured pharmacokinetic values, such as parent compound or metabolites in exhaled breath, blood, or urine, macromolecular adducts or degradation products of such adducts that appear in urine, can be used as biomarkers of exposure. To make quantitative assessments of the relationship of such markers to prior exposures, one must determine the rate of formation and removal (clearance) of the marker. From this information, one can predict the steady-state concentrations of the marker following various exposure scenarios. Also, with information on the rate of formation and removal of a marker, and the factors that influence those rates (such as gender, dose, repeated exposures, route of exposure, rate of exposure), one can develop a mathematical model that will describe the concentration of the marker under different exposure conditions. While the concentration of the marker cannot be used to indicate a unique exposure scenario, the marker can indicate the types of exposure regimens that would produce the indicated level of biomarker.

From a practical viewpoint, one cannot use human populations to determine the rate of formation and clearance of markers and the influence of various factors on those rates. Therefore, most toxicokinetic studies are conducted in animal models. From detailed studies in animals, mathematical models are derived

based on the animal toxicokinetic data, animal physiological data, and the physical/chemical properties of the compounds of interest (such as partition coefficients). The models, which are often referred to as physiologically-based pharmacokinetic models, can then be modified for humans based on human physiological data and metabolic studies conducted with human tissues in vitro. Such models must then be validated with limited studies in humans. This strategy has been discussed by Henderson and Belinsky [2].

A second strategy for the use of biomarkers to help describe prior exposures is to use a battery of biomarkers with differing half-lives [3]. Some biomarkers of exposure have half-lives of minutes or hours (volatile parent compound in exhaled breath, some blood or urinary metabolites); other biomarkers may be present for days or weeks (some DNA adducts, blood albumin adducts); while others may accumulate over longer periods of time due to longer half-lives (blood hemoglobin adducts, some DNA adducts, products of DNA repair in the urine). There are also differences in the fraction of the internal dose of a chemical that is converted to each type of biomarker. In general, some of the markers with shorter half-lives, such as urinary metabolites, represent large fractions of the internal dose, while macromolecular adducts, many of which have longer half-lives, represent only a small fraction of the dose. By combining knowledge of the half-lives of markers and the amount of marker formed relative to the total dose, one can obtain more information about a prior exposure using a battery of biomarkers than a single biomarker. For example, if multiple markers of a single chemical are determined in an individual, one should be able to distinguish between someone who has had a recent exposure, someone who is receiving an ongoing exposure and someone who was exposed repeatedly in the past but has had no recent exposures. If someone has had only a recent single exposure to a chemical, the shorter half-lived, more abundant biomarkers in the form of urinary metabolites should be readily detectable, but there should be very little of the longer half-lived, less abundant DNA adducts present. If the person has had an ongoing exposure for many years to the same chemical, there should be relatively high amounts of both the urinary metabolites and the DNA adducts. If the person was exposed some time ago, but not recently, then only the longer-lived DNA adducts or hemoglobin adducts may be detectable.

3. Strategies for use of biological markers of exposure to predict disease outcome

To be able to relate markers of exposure to health outcome, one needs to know which markers can be associated with the disease outcome and the degree of that association. That is, given a certain level of a biomarker of exposure, what is the probability of getting a disease? This query is certain to be made by participants in any study in which biomarkers of exposure are assayed in workers or in the general public. Currently, we have very little information on which to base an answer. The inability to use biological markers of exposure to predict health outcome represents a major gap in our knowledge and decreases the potential usefulness of the markers. What we need are valid markers of risk.

How can we improve our knowledge is this area? Perhaps the most fruitful area of research for discovering biomarkers of exposure that can be linked to disease outcome is the study of mechanisms of disease induction. One cannot define a marker of a 'biologically effective dose' unless one knows the mechanism by which the biological effect is induced. Likewise, one cannot know the earliest biological events that lead to a disease, unless one understands the mechanism of disease induction. Such mechanistic studies should provide the markers for steps that link the biological markers represented by traditional toxicokinetic measurements (left side of Fig. 1) and the biological markers represented by traditional clinical markers of disease (right side of Fig. 1).

In addition to knowledge of the mechanism of disease induction, one must define the quantitative relationship between the level of the marker and the probability of progression to an adverse health effect. Pharmaco- or toxicodynamic

modeling describing the kinetics of disease development is required in a manner similar to the toxicokinetic modeling used to describe the kinetics of internal dosimetry. For example, if one wants to use chemical-specific DNA adducts to predict cancer induction, the following pieces of information are required. First one must identify the various DNA adducts formed by the chemical. Then one must determine the biological half-lives of each adduct (How long will they be present before they are repaired?) and the mutagenic potential of each adduct (How much harm will the adducts cause if they are present?). If adducts are formed that have relatively long half-lives and high mutagenic potential, one can determine if the mutations induced by the adduct in in vitro studies are present in tumors induced by the chemical. Once enough is known about the disease induction to form an hypothesis for the process, one can use intervention studies, in which the proposed path to disease is blocked, to validate the path as the active disease-generating process. Finally, toxicodynamic models can be generated that describe the quantitative relationship between adduct levels and cancer induction. Such models will require knowledge of cellular dynamics involved in tumor formation.

The initial part of this approach can be illustrated by the studies of the mechanism of induction of liver tumors by vinyl chloride (VC) [4]. The four major DNA adducts formed by VC are the 7-(2-oxoethyl)-deoxyguanosine (OEdG), 3,N^4-ethenodeoxycytidine (EdC), 1,N^6-ethenodeoxyadenosine (EdA), and N^2,3-ethenodeoxyguanosine (EdG) [5]. The most abundant adduct formed is the OEdG, but this adduct has the shortest half-life of all the adducts. Also, the mutagenic potential of the OEdG adduct, as estimated by fidelity of DNA replication assays, is low [6], while the other adducts do induce mutations, especially the EdG adduct [7]. The relative amounts of the different adducts in the livers of VC-exposed adult rats were compared to the amounts in livers of similarly exposed newborn rats, which are more sensitive than adult rats to VC-induced hepatic tumors. The molar concentration of EdG was almost fourfold higher in the newborn livers than in the adult livers, while the other etheno adducts were similar in both age groups [8]. This information indicated that the EdG adduct was a better measure of the biologically effective dose (and of risk) than were the other adducts.

As in the case of relating biological markers to prior exposure, the relationship of the levels of markers to expected health outcomes can be studied first in animal models, as discussed above for studies on VC-induced tumors. But the models developed in animals must be validated by studies in humans (in vitro studies using human tissues, epidemiological studies). Studies reported by Qian et al. [9] are examples of how epidemiological studies can determine the most valid biological markers of risk for a disease. In a study of Chinese men exposed to aflatoxin in their diet, the investigators found that levels of urinary aflatoxin-N^7-guanine were a good predictor of the risk for hepatic cancer rather than other markers studied, including total aflatoxin-metabolites in urine and dietary intake of aflatoxin as assessed by questionnaires. Intervention studies, either in animals or in epidemiology studies, are also valuable in determining if the selected biological markers of risk are valid.

In conclusion, biomarkers can be valuable for reducing uncertainties in assessing risk for disease from chemical exposures. However, to make the markers more useful, more information is needed on the mechanisms of disease induction by chemicals; such studies will suggest the most appropriate biomarkers of risk for the disease. Much research effort will be required to establish quantitative relationships between the level of markers present and both the degree of prior exposure and the predictability of health outcome.

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Markers for immunotoxic effects in rodents and man

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Abstract

There is growing interest and concern in society about the capacity of chemicals to impair immune responses and trigger autoimmune disease. Methods to investigate chemical-induced immunosuppression have been developed and validated in the mouse and rat. Animal models that are suitable to investigate the ability of chemicals to induce autoimmune disease are virtually lacking. From the plethora of tests to assess immunity in man, panels of biomarkers to study immunotoxicity in humans have been proposed. Such studies in humans are considerably more complex than in animals as non-invasive tests are limited, responses in the population are heterogeneous, and exposure levels are often low. Human risk assessment is therefore mostly based on animal studies. As examples of compounds affecting biomarkers in animals and man, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and hexachlorobenzene are discussed.

Keywords: Immunotoxicology; Immunotoxicity testing; Immune suppression; Autoimmunity; Man; Rodents

1. Introduction

The functions of the immune system are the protection of the body from invading pathogens and to provide immune surveillance against arising tumor cells. It has a first line non-specific branch that can initiate effector reactions itself, and an acquired specific branch in which lymphocytes and antibodies carry the specificity of recognition and subsequent reactivity towards the antigen. The first line of innate immunity is constituted by phagocytic cells such as macrophages and polymorphonuclear granulocytes, or cytotoxic cells such as natural killer cells. After initial contact of the host with the pathogen, specific immune responses are induced. The hallmark of this second line of defense is specific recognition of determinants, so-called antigens or more in particular epitopes on these antigens, by receptors on the cell surface of B and T lymphocytes. Following recognition of these antigens lymphocytes proliferate and mature to plasma cells and produce antibodies (B cells), or differentiate to become cytotoxic T lymphocytes.

Immunotoxicology has been defined as the discipline concerned with the study of the events that can lead to undesired effects as a result of interaction of xenobiotics with the immune system. These undesired events may result as a consequence of: (1) a direct and/or indirect effect of the xenobiotic (and/or its biotransformation product) on the immune system, or (2) an immunologically based host response to the compound and/or its metabolite(s), or host an-

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tigens modified by the compound or its metabolites. When the immune system acts as a target of chemical insults, the result can be a decreased resistance to infection, certain forms of neoplasia, or immune disregulation/stimulation that can exacerbate or facilitate development of allergy or autoimmunity. Should the immune system respond to the antigenic specificity of the xenobiotic, toxicity can become manifest as allergies or autoimmune diseases.

Animal models to investigate chemical-induced immune suppression have been developed, and a number of these methods are validated. Several xenobiotics have been identified in such studies with laboratory animals to cause immunosuppression. The database on immune function disturbances in humans by environmental chemicals is limited. Immunotoxicity assessment in rodents, with subsequent extrapolation to man, forms the basis of human risk assessment. Methods to assess chemical-induced hypersensitivity in laboratory animals and in humans are available. The issue of chemical-induced allergy will not be discussed in this paper. Animal models that are suitable to investigate the ability of chemicals to induce autoimmune diseases are virtually lacking. Human data show that chemical agents, in particular drugs, can cause autoimmune diseases.

2. Immunotoxicity testing in laboratory animals

2.1. Immune suppression/stimulation

To predict induction of immune suppression by chemical exposure seems relatively easy as compared to induction of allergy and autoimmunity. Much work has been done on inclusion of general immune parameters in toxicity screening of chemicals. Two examples of tiered approaches are described. The first was developed at the National Institute of Public Health and the Environment, The Netherlands (RIVM) [1,2]. It is based on OECD guideline 407, and performed in the rat using at least 3 dose levels, i.e. one resulting in overt toxicity, one aimed at producing no toxicity, and one intermediate level. There is no immunization or challenge with an infectious agent. The first tier comprises general parameters including conventional hematology, serum immunoglobulin concentrations, bone marrow cellularity, weight and histology of lymphoid organs (thymus, spleen, lymph nodes, MALT), flow cytometric analysis of spleen cells, and possibly immunophenotyping of tissue sections. This approach has been used for the immunotoxic evaluation of pesticides [3].

In the OECD guideline 407 (adopted in 1981), the only parameters for the immune system were hematology including differential cell counting, in addition to histopathology of the spleen. In an evaluation that we made it appeared that this protocol was insufficient for identifying direct toxicity for the immune system [4]. Also, results of an international collaborative immunotoxicity study carried out by IPCS with the support of CEC showed that basic pathology investigations in the rat specified in OECD guideline 407 did not reveal the immune effects of azathioprine and cyclosporin A. The immunotoxic actions of these drugs could be detected provided the test was extended to include additional pathology parameters. An update of the OECD guideline has been proposed (revision of January 1994), and now includes: weight of spleen and thymus, and histopathology of these organs, in addition to lymph nodes, Peyer's patches, and bone marrow [5]. This update has been approved, and appears to be certainly an improvement over the earlier guideline, although even with this updated version, some immunotoxic chemicals may not be identified as such [6].

It should be noted that the array of tests proposed to be included in general testing of chemicals is aimed at flagging potential immunotoxicity. Once immunotoxicity has been flagged, further testing is required to confirm and extend the earlier findings. Further testing should include immune function testing. Besides confirming functional implications of the immunotoxicity identified, functional testing will likely provide information on no-adverse-effect levels, and are therefore valuable for the process of risk assessment. Functional tests that are especially valuable in this respect are host resistance assays. In such assays, animals that are exposed to the test chemicals are also exposed to pathogens. The

fate of the pathogen, and the pathology in the host associated with it, may serve as an indicator of the health implication of the immunotoxicity found for the test chemical. Pathogens used in these host resistance models are chosen in such a way that they are good models for human disease [7]. An interesting finding is that for certain compounds induction of immunotoxicity especially occurs during prenatal exposure.

The US National Toxicology Program has developed a tiered approach in mice that is linked closely with the standard protocol for subchronic oral toxicity and carcinogenicity studies [8]. Routinely exposure periods of 14–30 days have been used at dose levels that have no effect on body weight or other toxicological endpoints. Thus, compounds are identified for which the immune system represents the most sensitive target organ system. Tier one includes conventional hematology, lymphoid organ weight, cellularity and histology of the spleen, thymus and lymph nodes, ex vivo splenic IgM-antibody plaque-forming cell assay following sheep erythrocyte immunization, in vitro lymphocyte proliferation after stimulation with mitogens and allogeneic cells, and an in vitro assay for natural killer (NK) cell activity. In an adapted form of this approach, 51 different chemicals were evaluated, selected on the basis of structural relationships with previously identified immunotoxic chemicals [9]. The splenic IgM plaque-forming cell response and cell surface marker analyses showed the highest accuracy for identification of potential immunotoxicity. Lymphoid organ weight and histopathology proved to be comparatively insensitive parameters, likely due to the relatively low exposure levels in the top dose used.

2.2. Autoimmunity

Autoimmune disease occurs when an individual's immune system attacks own tissues or organs, resulting in functional impairment, inflammation and sometimes permanent damage. This disease with a multifactorial etiology results from the loss of immune tolerance to self-antigens. For the detection of the potential of compounds to exacerbate induced or genetically predisposed autoimmunity, animal models are available [10]. In induced models, a susceptible animal strain is immunized with a mixture of an adjuvant and an autoantigen isolated from the target organ. Examples are adjuvant arthritis, experimental encephalomyelitis and experimental uveitis in the Lewis strain rat. Examples of spontaneous models of autoimmune disease are the BB-rat and the NOD-mouse that develop autoimmune pancreatitis and subsequently diabetes, and the (NZBxNZW)F1 mouse or MRL/*lpr* mouse that develop pathology that resembles human systemic lupus erythematosus. These models are mainly used in the study of the pathogenesis of autoimmunity and the preclinical evaluation of immunosuppressive drugs. Very few studies have addressed the potential of these models for assessment of whether a xenobiotic exacerbates induced or congenital autoimmunity.

Induced autoimmunity can also result from the association of the compound with normal tisssue components such as to render them immunogenic. A variety of chemicals and drugs, in particular the latter, have been found to induce autoimmune-like responses [11]. For the detection of chemicals that produce this type of reaction, the popliteal lymph node assay (PLNA) in mice is a promising tool. The PLNA in mice [12] is based upon the hyperplasia (increase in weight) of lymph nodes in graft-versus-host reactions or pseudo-graft-versus-host reactions, and has been modified to assess the immunomodulatory potential of drugs. The test substance is injected s.c. into one hind footpad, and the contralateral side is either untreated or inoculated with vehicle alone. Comparison of PLN from both sides allows the effect of the test drug to be measured. Apart from differences in weight, histologic evidence of in vivo immunostimulatory activity can be discerned. These pseudo-graft-versus-host reactions with follicular hyperplasia have been documented in mice for drugs such as diphenylhydantoin, D-penicillamine and streptozotocin. The assay appears to be appropriate to recognize sensitizing, i.e. allergens and autoimmunogenic chemicals, as well as non-sensitizing immunostimulating compounds. The assay is not universally applicable, however, due

primarily to false-negative results. Some drugs, such as procainamide, are known to cause autoimmune-like responses in man, but are negative in the assay. This is presumably attributable to pharmacokinetic factors and may be minimized by testing metabolites. So far, over 60 compounds with documented adverse immune effects in humans were positive in the PLNA, i.e. caused an increase of PLN weight and cell numbers as compared to PLN of vehicle-injected controls [13,14]. Further research is required for the development of animal models suitable for testing the induction of autoimmune disease by chemicals.

3. Immunotoxicity assessment in man

It is obvious that many of the immunotoxic chemicals have been identified in rodent studies, as the database in man is less complete and often inconclusive. The most common design used in immunotoxicity research in man is the cross-sectional study [15]. In such a study, exposure status and immune function are measured at one point in time or over a short period of time. The immune function of 'exposed' subjects is compared to the immune function of 'non-exposed' subjects by the measurement of biomarkers. For this reason, proper identification of the exposed group should be done. This group should include subjects at the upper end of exposure. Where possible, the study should incorporate individual estimates or actual measurements of exposure. In the broadest sense biomarkers are measurements on biologic specimens that will elucidate the relationship between environmental exposures and human diseases, so that exposures and diseases can be prevented. In clinical medicine biomarkers have shown their value as a tool for the presence or absence of diseases or the course of the disease during therapeutic intervention. As such there are indicators for exposure, effect or susceptibility [16].

3.1. Markers of exposure

A biologic marker of exposure is a xenobiotic chemical or its metabolite or the product of an interaction between the chemical and some target cell or biomolecule. The most common markers of exposure are the concentration of the chemical in urine, blood or target organ or tissue. Immune-specific biomarkers of exposure are antibodies or positive skin tests to a particular chemical.

3.2. Markers of effect

A biomarker of effect is a measurable cellular or biochemical alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease. They range from markers measuring slight structural or functional changes to markers that are indicators of a subclinical stage of a disease or the manifestation of the disease itself. Functional changes in cells of the immune system by an immunotoxic chemical may be the first step in the process towards disease. For instance, longitudinal studies using asymptomatic individuals with low NK activity were found to have increased risk for upper respiratory infection and morbidity [17]. Immunosuppression may lead to more subtle changes in resistance to infections, such as influenza or common cold, rather than opportunistic infection. Also data in experimental animals indicate that small changes in immune function could increase the likelihood of disease [9].

Testing schemes for evaluation of individuals exposed to immunotoxicants are proposed among others by the Subcommittee on Immunotoxicology of the US National Research Council [16] and by a task group of the World Health Organization [10]. The panel proposed by the WHO is listed in Table 1, and is composed of assays that cover all major aspects of the immune system. Included are assays to test for the function of the humoral immunity, i.e. specific antibodies to tetanus or diphtheria (for which vaccination programs exist), and for the actual function of the cellular immunity using recall antigens. It should be mentioned that these tests were all developed for diagnostic purposes, but that in the context of immunotoxicity testing in man they are to be used in an epidemiological setting. This means that effects found in parameters between exposed group and control group

Table 1
Assays recommended for immunotoxicity assessment in man

(1) Complete blood count with differential
(2) Antibody-mediated immunity (one or more of following):
- Primary antibody response to protein antigen (e.g., epitope-labelled influenza vaccine)
- Immunoglobulin concentrations in serum (IgM, IgG, IgA, IgE)
- Secondary antibody response to protein antigen (diphtheria, tetanus or polio)
- Natural immunity to blood group antigens (e.g., anti-A, anti-B)

(3) Phenotypic analysis of lymphocytes by flow cytometry:
- Surface analysis of CD3, CD4, CD8, CD20

(4) Cellular immunity:
- DTH skin testing
- Primary DTH reaction to protein (KLH)
- Proliferation to recall antigens

(5) Autoantibodies and inflammation:
- C-reactive protein
- Autoantibody titers to nuclei (ANA), DNA, mitochondria and IgG (rheumatoid factor)
- IgE to allergens

(6) Measure of non-specific immunity:
- NK cell enumerations (CD56 or CD60) or cytolysis activity against K562
- Phagocytosis (NBT or chemiluminesce)

(7) Clinical chemistry screen
Proposal for all persons exposed to immunotoxicants

From [10].

may have a different biologic significance than an altered value in an individual person. Whereas a decrease in a single immune parameter in an individual person may not be indicative of increased susceptibility for disease, a subtle alteration in an immune biomarker in a population may indicate immunotoxicity.

3.3. Markers of susceptibility

Markers of susceptibility, also called effect modifiers, can act at any point along the exposure-disease continuum. Important sources of variability are genetic, endocrine, age-related and environmental factors. Over the last 2 decades it has become clear that many immunological disorders are linked to alleles of the major histocompatibility gene complex (MHC). The products of MHC alleles in humans (HLA antigens) have aroused interest at a clinical level as potential biomarkers of disease susceptibility. In some instances, there is a remarkable increase in relative risk of disease in individuals possessing particular alleles. Similar associations have been described in drug-induced immunological disorders. However, it should be noted that other genetic factors as well as environmental factors are also of importance. For susceptibility to immunotoxicity, there are not many biomarkers, other than in the case of the arylhydrocarbon (Ah) receptor, and sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Also stress of various types can effect the immune system and influence the susceptibility to and recovery from infectious, autoimmune and neoplastic diseases. Age-related variability is shown by the developing fetus which is more susceptible to immunotoxic effects than is the adult.

4. Bridging the gap between animal and humans

In experimental animals assessment of immunotoxicity is relatively easy, but it is difficult to extrapolate immunotoxic data from animals to humans. In humans, the tests that can be done are limited, the heterogeneity hampers identification of effects, and for obvious reasons, resistance to infections or tumors cannot be approached experimentally. In this sense, it should be noted that experimental studies in animals and studies in man are complimentary. Comparison of similar parameters in animal and

humans, including dose, toxicokinetics, (functional) effects on immune parameters, will provide insight in similarities and differences in sensitivity and mechanisms in animals and humans, and may help to extrapolate data from animals to humans that are not easily obtained in humans (e.g. resistance to infections).

Another promising tool for better extrapolation of animal data may be in vitro testing [9]. Sources for human material that are immunologically relevant, and that are accessible to study (in various degrees) are: blood; umbilical vein blood/endothelial cells; bone marrow; bronchoalveolar lavage; nasal lavage; skin biopsies; donor organs; cell lines. Most of such cell populations can also be derived from animals. Cell lines that are available include: macrophages; T cells (Th1 and Th2); B cells; granulocytes; keratinocytes; and thymus epithelial cells. For the cells from these sources many tests are available. These produce, among others, parameters such as viability, phagocytosis, respiratory burst, calcium influx, cytotoxicity, antigen presentation, antibody production, cytokine production, proliferation, apoptosis, and expression of cell surface markers.

It must be noted that in in vitro systems the complexity of the immune system as it exists in vivo is not present. This pertains to interactions between different cell types. For these reasons it is doubtful that in vitro testing will be very powerful in screening immunotoxicity of novel compounds in general. For prescreening for immunotoxicity of compounds in vitro testing may prove an adequate option. The strength of in vitro immunotoxicity testing lies, however, in studies aimed at unravelling mechanisms of immunotoxicity. Another approach is the use of severe combined immunodeficient (SCID) mice engrafted with human immune cells. This provides a special model to investigate immunotoxic chemicals.

The interpretation of animal or in vitro studies may be improved in cases where even limited human data are available using a 'parallelogram' approach. In general, if a parallelogram can be constructed in which data are available for 3 of the 4 cornerstones, it may be easier to predict the outcome at the remaining cornerstone. This approach may also be applicable in extrapolating deficits in immune function to increased susceptibility to infections in animal models as a means of interpreting the risk of disease in humans for which immune function data, but not infectious disease data, are available.

5. Examples of immunotoxicants

Examples of environmental immunotoxicants that induce immunosuppression are among others: polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs), oxidant gases, benzene, toluene, xylol, asbestos, dimethylnitrosamine, certain organotin compounds, lead, cadmium, mercury, benzo[*a*]pyrene. Many therapeutics exert immunotoxic activities, including alkylating agents, anti-inflammatory agents, anti-metabolites, estrogens, and opiates. Although uncommon, certain drugs and chemicals can occasionally trigger an autoimmune reaction. For example, occupational exposure to vinyl chloride, trichloroethylene, perchlorethylene, and epoxy resins may induce a scleroderma-like syndrome; procainamide, hydralazine and isoniazid can provoke drug-induced lupus in predisposed individuals; propylthiouracil occasionally causes systemic vasculitis associated with induction of anti-neutrophil cytoplasmic autoantibodies.

Some examples of chemicals that have a direct toxic action on the immune system will be dealt with below in more detail, in respect of the relevance of the findings for man.

5.1. PCBs, PCDFs and PCDDs

Numerous immunotoxicity studies, including the study of the mechanism of action, have been carried out with these chlorinated hydrocarbons. Studies with PCBs mostly comprise investigations with technical mixtures containing large numbers of congeners, including Ah receptor binding compounds. Most of the toxic responses of PCDDs, PCDFs and planar PCBs are thought to be mediated through the binding to this receptor, including the immunotoxic effects. Of the different PCDD and PCDF congeners, in

particular TCDD has been studied. These studies have shown that TCDD causes a wide variety of toxic effects, with a remarkable interspecies variation both in target organs and toxicity (e.g. LD_{50} values). Despite this variation, TCDD at sublethal doses causes thymic atrophy and immunotoxicity in all species investigated [18,19]. The atrophy of the thymus is histologically characterized by reduced cellularity of the thymic cortex. Results of immune function studies in mice, rats and guinea pigs indicate that TCDD suppresses cell-mediated immunity in a dose-related fashion. Parameters investigated include delayed-type hypersensitivity responses, rejection of skin transplants, graft-versus-host activity, and in vitro mitogen responses of lymphoid cells. This suppression appears to be an age-related phenomenon, as TCDD causes more severe immunotoxic effects after perinatal administration than after administration in adulthood. This may be associated with the fact that the thymus is active especially early in life. In the rat, perinatal exposure seems a prerequisite to produce immune suppression. Besides suppression of the cell-mediated immunity, TCDD can also impair humoral immunity. The effect on antibody synthesis after primary and secondary immunization are variable and require higher dose levels.

As a result of TCDD-induced immunosuppression, particularly of T cell-mediated responses, host resistance to various infectious agents is impaired [20]. Using different mouse strains and different treatment schedules TCDD has been shown to suppress the resistance to the infectious disease models *Salmonella bern*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, Herpes II, influenza and *Plasmodium yoelli* (malaria). Variable effects have been reported for the resistance to *Listeria monocytogenes*.

Immune suppression was not only seen in laboratory animals, but also in humans inadvertently exposed to PCBs, PCDFs and PCDDs [20]. Unequivocal immune alterations have been observed in Taiwanese residents following consumption of rice oil contaminated with PCBs and PCDFs. Exposure to these compounds caused acneiform skin lesions, pigmentation of skin and nails, liver damage and abnormal immune function. Serum IgM and IgA concentrations and the percentage of T lymphocytes in the peripheral blood were decreased [21]. The cell-mediated immune system was investigated by delayed-type hypersensitivity responses. The percentage of patients showing a positive skin test to streptokinase and streptodornase was significantly lower as compared to controls [22]. This suppression of cell-mediated immunity was reproduced in a follow-up study by tuberculin skin testing [23]. A disease similar to the Yu-Cheng poisoning in Taiwan occurred in Japan in 1968, the so-called Yusho disease. Yusho patients frequently suffered from respiratory infections. Serum IgA and IgM levels had considerably decreased during 2 years after the onset of the poisoning but returned to normal in most cases. Respiratory symptoms persisted for longer time periods [24]. From these investigations in humans it can be concluded that PCBs and related compounds cause immune alterations, particularly of the thymus-dependent immunity. The findings in man correlate with the findings in experimental animals, thus illustrating the relevance of studies in laboratory animals. However, as exposure data are virtually lacking for those individuals in which immune parameters were investigated, and because of the remarkable interspecies variation in toxicity, assessment of the risk of effects of PCDDs and related chemicals on the immune system of humans is not possible in quantitative terms. The transfer of human thymus to SCID mice provides an opportunity to study the sensitivity of the human thymus to these compounds, in particular TCDD.

SCID mice have an autosomal recessive defect that impairs the rearrangement of antigen receptor genes in both T and B lymphocyte progenitors [25], and as a result they lack functional T and B cells. SCID mice engrafted with human fetal thymus and liver tissue (SCID-hu mice) have been shown to sustain human T cell differentiation in the human thymus grafts. The model provides the opportunity to examine the sensitivity of the human thymus to thymotoxic chemicals like TCDD [26]. Wistar rats and SCID-hu mice were exposed once to 0, 1, 5 or 25 μg

TCDD/kg body weight. The relative size of the cortex, evaluated on day 4 after treatment, was decreased at 25 μg/kg both in rat thymus and the grafted human thymus. SCID-ra mice (engrafted with fetal rat thymus and liver) were used as controls, and showed comparable effects. TCDD tissue concentrations in the normal rat thymus and the grafted human thymus were similar. The study indicates that the human thymus and the Wistar rat thymus display a similar sensitivity to TCDD.

5.2. Hexachlorobenzene (HCB)

HCB is a highly persistent environmental chemical that has been used in the past as a fungicide. Presently, emission in the environment may occur as a result of the use of HCB as a chemical intermediate or as a byproduct in chemical processes. It is an immunotoxic compound, with different effects in rats and mice [27]. In rats, prominent changes following dietary exposure include elevated IgM levels and an increase in the weights of the spleen and lymph nodes. Histopathologically, the spleen shows hyperplasia of B lymphocytes in the marginal zone and follicles, while lymph nodes show an increase in proportions of high endothelial venules, indicative of activation. High endothelial-like venules are induced in the lung, as are accumulations of macrophages. Functional tests revealed an increase in cell-mediated immunity, as measured by delayed-type hypersensitivity (DTH) reactions, and a notable increase in primary and secondary antibody response to tetanus toxoid [27]. Stimulation of humoral and cell-mediated immunity occurred even at dietary levels as low as 4 mg/kg following pre- and postnatal exposure; at such a dose conventional parameters for hepatotoxicity were unaltered. Therefore, the developing immune system of the rat seems particularly vulnerable to the immunotoxic action of HCB.

More recent studies indicate that HCB might cause autoimmune-like effects in the rat. Wistar rats treated with HCB had elevated levels of IgM, but not IgG, against the autoantigens single-stranded DNA, native DNA, rat IgG (representing rheumatoid factor), and bromelain-treated mouse erythrocytes (that expose phosphatidylcholine as a major autoantigen). It has been suggested that HCB activates a recently described B cell subset committed to the production of these autoantibodies [28]. The role of these autoantibodies is still a matter of controversy. Increased levels have been associated with various systemic autoimmune diseases, but a protective role of these autoantibodies against development of autoimmune disease has been postulated as well. Interesting in this respect are the observations that HCB had quite opposite effects in 2 different models of autoimmune disease in the Lewis rat. HCB treatment severely potentiated allergic encephalomyelitis elicited by immunization with myelin in complete Freund's adjuvant, while it strongly inhibited the development of arthritic lesions elicited by complete Freund's adjuvant as such [29].

The chemical HCB has been the subject of intense research following the accidental poisoning in Turkey from 1955 to 1959: it has been estimated that 4000 people developed porphyria due to the ingestion of wheat that was treated with the fungicide HCB [30]. Clinically, patients developed skin lesions that have been attributed to the the toxicity of photochemically activated cutaneous porphyrins. In a follow-up study of 204 patients, 20–30 years later, dermatological and other abnormalities, such as painless arthritis, still persisted [31]. Regarding the mechanism of HCB-induced hepatic porphyria, an oxidative metabolite and not the parent compound has been found to be responsible for the porphyrinogenic action. In rats treated with the combination of HCB and triacetyloleandomycin (TAO, a selective inhibitor of cytochrome P450IIIA), porphyria was greatly reduced [32,33]. Remarkably, combined treatment with HCB and TAO did not substantially affect incidence and severity of skin lesions. In addition, TAO did not influence the immunomodulatory effects of HCB, including the formation of autoantibodies. From these findings it has been suggested that an immunological component at least partially underlies the HCB-induced skin lesions in the rat and possibly in man [34]. Similarly, an autoimmune etiology is conceivable

as to other symptoms in HCB-poisoned patients in Turkey such as enlarged thyroid and painless arthritis [31]. This certainly needs future research.

6. Conclusions

Studies in rodents have provided a great number of markers to obtain meaningful information on potential immunotoxicity as it pertains to immunosuppression and reduced resistance to infectious diseases. Several xenobiotics have been identified in such studies with laboratory animals to impair immune responses and host resistance. In humans, the database on chemical-induced immunosuppression is limited, as the use of markers of immunotoxicity has received little attention in clinical and epidemiological studies. Such studies have not been performed frequently, and their interpretation often does not permit unequivocal conclusions to be drawn, due for instance to the uncontrolled nature of exposure. Incidental information is promising in that in general terms human data confirm the data gained with experimental animals. Immunotoxicity assessment in rodents forms therefore the basis for human risk assessment.

Animal models are currently available to detect the potential of compounds to exacerbate induced or genetically predisposed autoimmunity, but are seldomly used in immunotoxicity studies. Models to investigate the ability of chemicals to induce autoimmunity, as result from an immune response to own proteins modified by the chemical, are virtually limited to the PLNA. As human data show that chemical agents, in particular drugs, can cause autoimmune diseases, new models should be developed.

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Glutathione peroxidase and oxidative stress

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Abstract

In this study, overexpression of the cDNA for the major cytoplasmic glutathione peroxidase isoenzyme, GSH Peroxidase 1 (GSHPx-1), in human MCF-7 breast cancer cells has been shown to significantly increase the tolerance of these cells to oxidative stress produced by hydrogen peroxide or by the redox cycling of the quinone-containing anticancer agent doxorubicin. These experiments suggest that intracellular detoxification of hydrogen peroxide by the glutathione-glutathione peroxidase cycle plays a critical role in resistance against oxidant stress produced by xenobiotics.

Keywords: Glutathione peroxidase; Anthracyclines; Doxorubicin; Hydrogen peroxide; Selenium

1. Introduction

Glutathione-dependent pathways for intracellular detoxification play an important role in modulating the growth inhibitory effects of xenobiotics, including the anticancer quinone doxorubicin [1,2]. Of the enzymatic detoxification pathways, the four well-characterized, selenium-dependent glutathione peroxidases (GSHPx) play an essential part in the metabolism of H_2O_2, fatty acid hydroperoxides, and phospholipid hydroperoxides in mammalian cells [3–6]. Of these isoenzymes, the cytoplasmic species, GSHPx-1, has been most clearly linked to alterations in the cytotoxicity of the anthracycline quinone doxorubicin [7–11]. To examine the role of GSHPx-1 in the cytotoxicity produced by oxidative stress, the human GSHPx-1 cDNA was expressed in MCF-7 breast carcinoma cells, a human tumor line which possesses barely detectable levels of glutathione peroxidase prior to transfection [4]. Under conditions that allowed an over 25-fold increase in glutathione peroxidase expression, the cytotoxicity of hydrogen peroxide or doxorubicin was significantly decreased.

2. Experimental procedures

2.1. Materials

Doxorubicin hydrochloride was purchased from Adria Laboratories (Wilmington, DE); Reduced glutathione (GSH), glutathione reductase type III, 5′-5′-dithiobis-(2-nitrobenzoic acid), 1-chloro-2,4-dinitrobenzene (CDNB), G418, and NADPH, were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (30% solution) was supplied by Mallinckrodt Inc. (St. Louis, MO). DMEM/F12 minimal es-

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sential medium and heat-inactivated fetal calf serum (FCS) were purchased from GIBCO (Grand Island, NY).

2.2. Methods

MCF-7 human breast carcinoma cells, obtained from the American Type Culture Collection (Rockville, MD), were initially cultured in DMEM/F12 medium with 5% FCS at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cloning and expression of the human GSHPx-1 cDNA in MCF-7 cells has been described previously [4]. MCF-7H6, a transfectant expressing high levels of enzymatic activity, as well as the parental MCF-7 line were adapted to growth in 0.5% FCS containing 5 μg/ml each of insulin and transferrin and 400 μg/ml of G418 to which 30 nM selenium was added. Growth inhibition was determined with cells in exponential growth phase (96 h after plating) by addition of 50 μl of the compound of interest (or medium for controls) to the tissue culture flasks in triplicate for a 1-h period of exposure at 37°C. After drug exposure, the medium was removed and cells were washed in phosphate-buffered physiologic saline (PBS); cells were then harvested by treatment with 4 ml of a mixture containing 0.05% (w/v) crystalline trypsin and 680 μM EDTA for 1 min at 37°C. The cell suspension was then removed from the tissue culture flasks, passed through a 5-ml pipette, washed in PBS, and counted in a hemocytometer. Cell viability (in all cases >90%) was measured by exclusion of 0.1% trypan blue dye. Cells were then replated in 25-cm^2 tissue culture flasks in duplicate in DMEM/F12 medium with 5% FCS without selenium and cultured at 37°C. Growth inhibition was determined 96 h after exposure by comparison of cell numbers in treated versus control flasks; the data have been expressed as the percentage of growth of treated cells compared to that of cells exposed to medium alone. The activities of antioxidant enzymes in MCF-7 or MCF-7H6 cells were measured as described previously [1]. Data were analyzed with the two-tailed Student's *t*-test for independent means (NS, $P > 0.05$ [12]). Data are expressed as the mean ±1 S.E.

3. Results

3.1. Effect of GSHPx-1 overexpression on antioxidant levels in MCF-7 cells

Expression of the cytosolic glutathione peroxidase cDNA in MCF-7 cells resulted in an approximate 25-fold increase in GSHPx activity (Table 1). Furthermore, overexpression of glutathione peroxidase did not lead to any change in the expression of catalase or glutathione *S*-transferase (GST) activity in the cytoplasmic compartment of the MCF-7 cells. Levels of reduced glutathione were also unchanged in the transfectants compared to the parental line (data not shown).

3.2. Effect of GSHPx-1 overexpression on oxidant cytotoxicity in MCF-7 cells

To evaluate the role of glutathione peroxidase in detoxifying peroxides, MCF-7 cell transfectants were exposed to hydrogen peroxide as well as to the redox cycling anticancer quinone doxorubicin. As shown in Table 2, overexpression of

Table 1
Antioxidant enzyme levels in MCF-7 GSHPx-1 transfectants

Enzyme activity (nmol/min/mg)	MCF-7	MCF-7H6
GSHPx-1	1.8 ± 0.5	55.8 ± 3.1*
GST	2.1 ± 0.4	3.4 ± 0.9
Catalase	1900 ± 340	1700 ± 400

* $P < 0.01$ compared to MCF-7.

Table 2
GSHPx-1 expression and oxidant cytotoxicity

Oxidant	MCF-7	MCF-7H6
H_2O_2 (100 μM)	34 ± 2[a]	79 ± 2*
Doxorubicin (1 μM)	43 ± 8	83 ± 4*

[a] Cell growth as per cent of control ±S.E.
* $P < 0.05$ versus MCF-7.

GSHPx-1 significantly decreased the growth-inhibitory effects of these oxidants.

4. Discussion

Although the glutathione peroxidase isoenzymes are present in all mammalian tissues, the role of these selenium-dependent enzymes in protecting cells against oxidant stress has been questioned [13]. This controversy has, in part, been based on the lack of a specific glutathione peroxidase inhibitor, which has made selenium deprivation the only available means to reliably alter intracellular GSHPx activity [2]. The availability of the GSHPx-1 transfectants that have recently been described by our laboratory has allowed studies of the antioxidant role of the cytoplasmic enzyme to proceed.

When MCF-7 human breast cancer cells are transfected with a human GSHPx-1 cDNA, selenium-dependent glutathione peroxidase activity increases over 25-fold. This occurred without altering the growth rate of the cells or the expression of other components of the intracellular antioxidant milieu (catalase, glutathione, or GST). The transfectants also became relatively resistant to the growth-inhibitory effects of hydrogen peroxide or doxorubicin, suggesting that GSHPx activity was critical for the protection against oxidant stress that was observed.

However, the resistance demonstrated was not complete. This observation may be related to the fact that the oxidants we have chosen to study produce potentially-lethal damage at the level of the cell surface, as well as intracellularly [14]. Furthermore, these studies do not address the mechanism(s) involved in producing the level of resistance that was observed; thus, additional experiments will be required to determine the functions critical for cell growth that have been spared by enhancement of peroxide detoxification. Overall, however, these investigations do suggest that the GSHPx system *itself* may play an essential role in modulating the growth-related phenomena which are regulated by cellular peroxide tone.

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Amplification of glutamate-induced oxidative stress

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Abstract

Glutamate is a ubiquitous neurotransmitter which causes excess neuronal excitotoxicity and neurodegenerative insults such as stroke, trauma and seizures. A salient feature of the activation of glutamate receptors is the induction of oxidative burst. Moreover, glutamate stimulates Ca^{2+} influx and translocates protein kinase C (PKC). PKC mediates cellular processes mediated via phosphorylations which may be essential for oxidative burst in many cells. Subsequent oxidative stress may be a causal factor of neurodegenerative diseases. Increased glutamate release and oxidative burst may thus both be essential in the cascade of events leading to neuronal damage. Glutamate may also mediate neurotoxic effects of environmental toxic agents such as lead which amplify glutamate excitotoxicity. In these interactions, excessive activation of glutamate receptors and oxidative burst may converge into a common pathway leading to cell death through a cascade involving PKC or other proteins important in oxidative burst in neurons.

Keywords: Neuronal cells; Glutamate-induced cell activation; Lead; Protein kinase C; Oxidative stress; Cell injury

1. Introduction

Glutamate is the most important excitatory neurotransmitter in the brain. Human brain contains about 10^{10} neurons each exhibiting approximately 1000 synapses. About 90% of these 10^{13} synapses utilize amino acids as their neurotransmitter. Among the amino acids glutamate is the most predominant one and is released by an estimated 40% of all synapses [1,2]. Co-existence and release, association in neuronal events, and coupling mechanisms of glutamate, aspartate, and acetylcholine (ACh), have recently evoked remarkable interest in events related to excitatory neuronal damage and death [3–7]. Glutamate-induced activation of ionotropic glutaminergic receptors is associated with influx of Ca^{2+}, and excessive activation of these receptors seems to cause subsequent oxidative burst. Both glutamate and oxidative stress have been implicated in stroke, trauma and seizures [1].

In addition to overt toxic insults, recent observations suggest that glutamate may have a widespread role in the central nervous system (CNS) also in mediating neurotoxic effects of environmental toxic agents. Markovac and Goldstein have shown that lead at picomolar concentrations stimulates protein kinase C (PKC) in acellular systems [8] and that at micromolar concentrations it activates PKC in brain microvessels [9]. PKC, in turn, has a key role in

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the production of reactive oxygen species in immunological cells by phosphorylating NADPH oxidase which produces superoxide anion ($^{\cdot}O_2^-$) [10,11]. Glutamate has been shown to induce the production of reactive oxygen species in neuronal preparations [12], and Cid and Ortega [13] have provided evidence that PKC may have an important role in glutamate-induced oxidative burst.

2. Oxidative stress and cerebral insults

Oxidative stress in the CNS elevates free intracellular Ca^{2+}, increases the release of excitatory amino acids, mainly glutamate, may increase lipid peroxidation, and depletes neuronal glutathione (GSH) [7,14]. Oxidative stress results from the formation of reactive oxygen species, $^{\cdot}O_2^-$, the hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2). They are generated as byproducts of normal and aberrant neuronal metabolism that utilizes molecular oxygen [14]. Recent observations indicate that the activation of glutamate-activated cation channels may be intrinsically involved in neuronal oxidative stress. These 2 processes may be interrelated and sequential processes which jointly form a common pathway leading to oxidative damage of neurons [1].

Relevance of both glutaminergic agonists and ACh have been implicated in a number of cerebral disorders and both induce seizures and neuronal damage in rodents [1,7,15–18].

Cholinergic convulsions and associated brain damage can be prevented by pretreating animals with a cholinergic antagonist atropine. Atropine is, however, without an effect if given after the onset of convulsions. By utilizing specific *N*-methyl-D-aspartate (NMDA) receptor agonists both cholinergic and kainic acid-induced convulsions and associated neuronal damage can, however, be prevented [17,19] indicating that neurotransmitters other than ACh are responsible also for the final deleterious outcomes of excessive cholinergic brain stimulation. Moreover, increased glutamate release has been found after the induction of convulsions in rats with kainic acid or soman [15]. These findings have been substantiated by observations of Docherty et al. [4] which show that cholinergic stimulation of neurons is associated with a co-release of excitatory amino acids glutamate and aspartate.

3. Glutamate-induced cell excitation

Excessive release of glutamate in the CNS is associated with elevated cellular Ca^{2+} levels ($[Ca^{2+}]_i$) and neuronal damage [1]. Naarala et al. [20] showed $[Ca^{2+}]_i$ elevation in human SH-SY5Y neuroblastoma cells by stimulating NMDA, kainate, and α-amino-3-hydroxy-5-methyl-4-isoxalolpropionic acid (AMPA) glutamate receptors. Stimulation of metabotropic glutaminergic receptor with *trans*-1-amino-cyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) also elevated $[Ca^{2+}]_i$ in these cells. However, in isolated synaptoneurosomes, increased production of reactive oxygen species could be induced only by agonists of ionotropic glutamate receptors, notably NMDA, kainate, and AMPA, and not by an agonist of the metabotropic glutamate receptor [12]. On the contrary, Lafon-Cazal et al. [21] demonstrated that stimulation of NMDA receptors, but not of kainate receptors or voltage-sensitive Ca^{2+}-channels, in cultured cerebellar granule cells increased the production of $^{\cdot}O_2^-$. The production of $^{\cdot}O_2^-$ was due to increase of $[Ca^{2+}]_i$ and facilitated metabolism of arachidonic acid as a consequence of phospholipase A_2 activation.

The role of PKC in glutamate-induced oxidative burst has remained open but Cid and Ortega [13] observed that both glutamate and its structural analogue kainate evoke a dose-dependent increase in the maximal number of binding sites for [^{3}H]phorbol 12,13-dibutyrate (PDBu) in intact cultured chick cerebellar Bergman glial cells. This indicates translocation of PKC to the membrane. Glutamate and kainate responses were mediated via AMPA/kainate receptors. These findings are consistent with the observations of Bondy and Lee [12] and in agreement with a possible role of PKC in glutamate-induced oxidative burst in neuronal cells. However, glutamate-induced translocation of PKC via AMPA/kainate receptors is contrary to findings of Lafon-

Cazal et al. [21]. These discrepancies may indicate that glutamate-induced oxidative burst is mediated via different glutamate receptor subtypes in different neuronal preparations and that the role of PKC in these events differ, possibly due to differences in the expression of PKC isoenzymes.

The potential of glutamate to produce oxidative stress in neurons is of particular interest because the release of glutamate from synaptosomes further amplifies the production of reactive oxygen species thereby causing amplification of glutamate-induced neuronal damage [22]. In this context it is important to note that a 10 000-fold gradient exists between glutamate concentration in the extracellular space in cerebral spinal fluid (0.3 μM) and the average brain parenchyma (3000 μM). In the synaptic vesicles, the concentration of glutamate is even higher, about 10 mM. The primary mechanism for clearing extracellular glutamate is a family of Na^+-dependent high-affinity transporters on glutaminergic neuronal processes and glial cells. Clearing of glutamate from the extracellular space reduces glutamate excitotoxicity to adjacent cells [1,2]. However, insults evoking an increased release of glutamate or the production of reactive oxygen species may initiate a vicious cycle leading to widespread nervous destruction. Savolainen and coworkers [7,18] have suggested that convulsions and associated brain damage induced by cholinergic stimulation may utilize a common pathway also active in glutaminergic convulsions and other insults in which glutamate is involved [15]. In these events both the release of glutamate and increased production of reactive oxygen species may play a central role.

Present data suggest that glutamate and oxidative stress may play an important role also in neuronal apoptosis in a variety of brain conditions [23]. Sustained increases in $[Ca^{2+}]_i$ have been shown to be associated in cellular events leading to apoptosis but it is not clear whether $[Ca^{2+}]_i$ is one of the causal factors or an epiphenomenon that occurs simultaneously with apoptosis [1,23]. In support of the potential role of apoptosis in glutamate toxicity is the inhibition of oxidative stress-induced neuronal death in neurons overexpressing *bcl*-2 proto-oncogene preventing apoptosis [24,25]. Moreover, Mitchell et al. [26] demonstrated that reserpine-induced apoptosis in striatal projection neurons could be prevented by glutamate antagonists in vivo. The issue on glutamate-induced apoptosis in neuronal cells is, however, controversial because the activation of metabotropic glutamate receptors have been shown to prevent the low K^+-induced apoptosis in cultured granule cells [27].

4. Lead-induced neuronal damage

Exposure to inorganic or organic lead may produce clinically definable encephalopathy and neuropathy that depend on age, route of administration, and dose [28]. High doses of lead may produce either severe CNS symptoms such as ataxia, convulsions or coma, or lesser CNS deficits including learning disorders, hyperactivity and headache in humans [29]. Moreover, Cohn and Cory-Slechta [30] have recently observed in rats that exposure to low levels of lead in drinking water markedly amplified NMDA-induced discrimination learning in rats. In support of this finding is the observation by Guilarte et al. [31] that both prenatal and postnatal exposure of rats to low levels of lead increases the binding of [^{3}H]MK-801 to NMDA receptors in adult rat forebrain.

Even if the effects of lead have been thoroughly described, the biochemical and molecular mechanisms of lead toxicity are poorly understood. Recent data suggest that some of the effects of lead may be due to its interference with Ca^{2+}-mediated cellular processes such as calmodulin and calmodulin-sensitive enzymes, e.g. activation of phosphodiesterase responsible for the hydrolysis of cyclic AMP [8,32–35]. Effects of lead may be directed also to the release of neurotransmitters, e.g. norepinephrine, dopamine, ACh, or GABA [8,32–34], or the activation of protein kinases [8,9,34,36,37]. Lead may, in fact, activate PKC by mimicking Ca^{2+} [8,9,37], and this may result in the production of reactive oxygen metabolites [10]. Both organic and inorganic lead increase lipid peroxidation and the production of reactive oxygen species in a num-

ber of different cell types [38,39]. In rats, lead accumulates especially in the cerebral cortex, and decreases the concentrations of phospholipids in those brain areas in which the lead content is the highest. These changes are associated with increased rate of lipid peroxidation in rat brain [40].

Lead increased the binding of inositol-1,4,5-trisphosphate ($InsP_3$) to its receptor on the intracellular Ca^{2+} vesicles in rat cerebral microsomes whereas $InsP_3$-induced $[Ca^{2+}]_i$ elevation was inhibited. Reuptake of Ca^{2+} into the microsomes as well as microsomal Ca^{2+}-pump were also inhibited by lead [41]. Thus, lead may modulate inositol lipid-mediated cellular signalling implicated in the actions of metabotropic glutamate receptors [1,5].

5. Amplification of glutamate-induced oxidative stress: the role of PKC

Production of glutamate-induced reactive oxygen species has been demonstrated convincingly in a number of recent papers [12,21,39,42,43]. However, the possible mechanisms behind this effect began to unfold after the observation that glutamate stimulates PDBu binding in neuronal cells. Both glutamate and kainate evoked a dose-dependent increase in PDBu-binding in intact neuronal cells indicating translocation of PKC from the cytosol to the membrane. The effect was mediated through the AMPA/kainate receptors. This finding brought up the possibility that PKC may play an important role in glutamate-induced neuronal processes such as oxidative burst.

Naarala et al. [20] have shown that human SH-SY5Y neuroblastoma cells express both ionotropic NMDA, kainate and AMPA receptors and metabolic glutamate receptors rendering these cells suitable for studies on mechanisms of glutamate-induced excitation. Utilizing these cells Naarala et al. [44] were able to demonstrate glutamate-induced oxidative burst in these cells which was dramatically amplified by lead. Glutamate-induced production of reactive oxygen species could only be demonstrated when neuronal GSH was depleted by diethyl maleate indicating an effective GSH defence against oxidative stress in these cells. Moreover, glutamate-induced production of reactive oxygen species could be completely blocked by a competitive inhibitor of PKC, Ro 31-7549 [44,45]. Thus, PKC was evidently involved in glutamate-induced oxidative burst. The finding that glutamate increases PDBu-binding further substantiates the conclusion on the role of PKC in glutamate-induced oxidative burst [13]. The ability of lead to stimulate PKC in aqueous solutions at picomolar concentrations [8] and in brain microvessels at micromolar concentrations [9] adds an interesting dimension to the lead-glutamate interaction. Long et al. [37] have also confirmed, utilizing ^{19}F NMR spectroscopy, the ability of picomolar lead concentrations to activate brain PKC. Laterra et al. [36] observed that lead inhibits differentiation of brain microvessel endothelial cells through an effect on PKC confirming the importance of PKC as a target for lead action.

A likely explanation for the findings of Naarala et al. [44] is that lead amplifies glutamate-induced oxidative burst by mimicking Ca^{2+} [8,9], and thereby activates PKC. Thus, glutamate may increase both translocation and activation of PKC which is further potentiated by a direct activation of PKC by lead. These events may together explain the eventual oxidative burst, decreased neuronal GSH, and increased cytotoxicity.

To further explore the role of PKC in oxidative stress, human neuroblastoma cells were treated with lead and potent PKC inhibitors, Ro 31-7549 or Ro 31-8220, with low IC_{50} values for brain PKC of 0.08 and 0.01 μM, respectively [45]. These compounds effectively inhibit PKC-induced phosphorylations. Also lead and PKC inhibitors jointly increased the production of reactive oxygen species in human neuroblastoma cells even if the compounds were inactive alone. PKC inhibition by Ro 31-8220 induced, however, translocation of PKC to the membrane. Lead and PKC inhibition also decreased neuronal GSH and increased cytotoxicity in human neuroblastoma cells (Naarala et al., unpublished).

Unphosphorylated PKC target enzymes may be sensitive to the activating effects of lead in analogy with PKC which is potently activated by lead [8,37]. Enzymes not being phosphorylated due to PKC inhibition may contribute to the production of reactive oxygen species when exposed to lead. Furthermore, a PKC inhibitor may activate kinases which do not depend on the formation of diacylglycerol, a second messenger in the inositol lipid signalling pathway, and PKC activity [10]. A PKC inhibitor may also affect the production of reactive oxygen species through an effect on cell membrane phospholipid metabolism [46]. However, lead-induced direct activation of PKC [8,37] is not relevant in the present interaction of lead with Ro 31-7549 [45]. Figs. 1 and 2 present proposed effects of presynaptically released glutamate in a postsynaptic neuron, and interactions of lead and PKC inhibitors, Ro 31-7549 or Ro 31-8220, with glutamate-evoked postsynaptic responses.

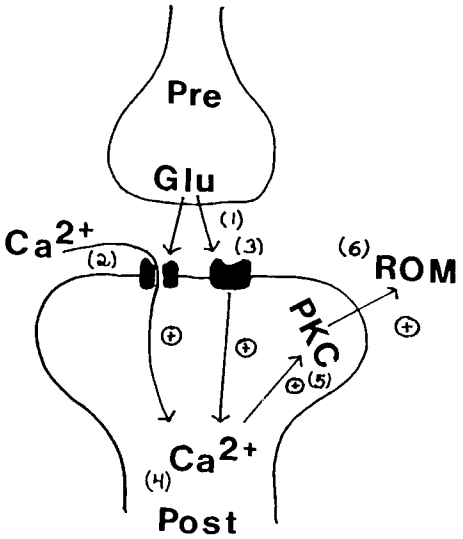


Fig. 1. Glutamate and the production of reactive oxygen metabolites (ROM). Upon neuronal stimulation (1) glutamate is released from the presynaptic neuron (Pre) into synaptic cleft; glutamate binds in the postsynaptic cell (Post) either to (2) ionotropic (NMDA, kainate, AMPA) glutamate receptors increasing the influx of Ca^{2+} or to (3) metabotropic glutamate receptors which through activation of a G-protein and phospholipase C increase the production of $InsP_3$ and increase the release of Ca^{2+} from non-mitochondrial Ca^{2+} vesicles; (4) levels of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) rise upon glutamate receptor stimulation which causes (5) translocation and activation of PKC and ultimately (6) increased production of ROM.

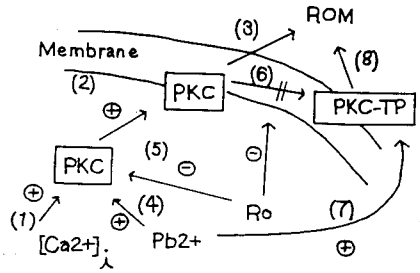


Fig. 2. Interactions of calcium, lead, and a PKC inhibitor, Ro 31-7549 or Ro 31-8220 (Ro), with PKC and its putative target proteins (PKC-TP). (1) Elevation of $[Ca^{2+}]_i$ causes (2) translocation and activation of PKC and (3) subsequent production of ROM; (4) lead also activates PKC, and increases (3) the production of ROM; (5) Ro inhibits PKC but increases simultaneously the translocation of PKC to the membrane; (6) Ro-induced inhibition of PKC prevents PKC-mediated phosphorylation of PKC-TP some of which may increase the production of ROM in an unphosphorylated form; (7) lead may activate putative PKC-TP to (8) produce ROM.

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Function and activation of the transcription factor NF-κB in the response to toxins and pathogens

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Abstract

The ubiquitous transcription factor NF-κB regulates the expression of a plethora of genes. In most cell types the dimeric DNA-binding form of the transcription factor is retained in the cytoplasm by physical association with the inhibitory IκB molecules. Five distinct DNA-binding subunits have so far been identified in vertebrates, which share a N-terminal homology region responsible for DNA-binding and dimerization. Exposure of cells to a variety of pathogenic and/or toxic agents leads to the generation of hydrogen peroxide and the inducible phosphorylation of IκB, which is a prerequisite for its proteolytic degradation. The DNA-binding subunits can subsequently enter the nucleus, bind to their cognate DNA and activate transcription.

Keywords: Transcription factor NF-κB; Signal transduction; Oxygen radicals

1. Molecular biology of the DNA-binding subunits

Purification of NF-κB using sequence-specific DNA affinity chromatography revealed 2 proteins of approximately 50 and 65 kDa molecular weight referred to as p50 and p65 [1]. These proteins were shown to contact the decameric κB motif (consensus: 5′-GGGPuNNPyPyCC-3′) as a heterodimer. Binding sites for NF-κB are found in the enhancer and promoter regions of many cellular and viral target genes, including transcription factors, viruses, immunoreceptors, cell adhesion molecules, cytokines, hematopoetic growth factors and acute phase proteins [2]. Molecular cloning of the p50 and the p65 DNA-binding subunit revealed that these proteins share a homology domain of approximately 300 amino acids in their N-terminus. This domain is also contained in the oncogene *v-rel* from the turkey retrovirus REV-T, as well as its cellular homologue *c-rel* and in the *Drosophila melanogaster* proteins dorsal and Dif. PCR cloning and low-stringency hybridization revealed p52 and RelB as additional members of the NF-κB/Rel family [3,4]. The homology region is referred to as NRD domain, since it is shared by NF-κB, Rel and Dorsal. The NRD domain is necessary for DNA-binding, dimerization, nuclear location and the interaction with IκB proteins. The majority of the 5 different subunits from vertebrates can homo- and dimerize with each other, but the most frequently detected form of NF-κB consists of a heterodimer between p50 and p65. Studies employing trans-dominant mutants and domain-swop experiments located the amino acids responsible for DNA-binding and recognition to the N-terminal half of the NRD domain. The

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dimerization domain was mapped to the more C-terminal half of the homology region. These biochemical and genetic data are completely consistent with the crystal structure which was obtained for the p50 subunit. A cocrystal between a p50 dimer and DNA revealed that the NRD domain can be further divided into 2 distinct subdomains, similar to those in the immunoglobulin superfamily. The DNA is contacted by the N-terminal specificity domain, that interacts with the DNA bases. This subdomain is composed of a 9-strand antiparallel β-barrel, from which emanate several loops important for DNA recognition at the sugar phosphate backbone. The more C-terminal dimerization domain forms a dimerization interface between β-sheets using residues strongly conserved between the members of the NRD family [5,6]. The nuclear location signal is located at the C-terminal end of the NRD domain and contains a short stretch of 4 or 5 basic amino acids. The p50 and p52 proteins are synthesized as large precursor molecules of 105 and 100 kDa molecular weight, respectively. The C-terminal halves of these precursors contain a repeated sequence motif (the so-called ANK repeat) discovered earlier in the SWI6 protein from yeast and also found in the IκB proteins. Both precursor molecules are located in the cytoplasm and are unable to bind to DNA. The p50 (and presumably also the p52) protein is generated by proteolytical processing from the precursor molecule using the ATP-dependent ubiquitin-proteasome pathway [7].

In contrast to p50 and p52 proteins the 3 other members of the NRD family are transcriptionally active. C-terminally located transactivation domains have been found in c-Rel, RelB and p65 proteins. Structural and functional analysis revealed that the activation domains of p65 and c-Rel belong to the class of acidic activators and directly contact components of the basal transcription complex [8]. Excess p50 can downregulate gene expression by occupying NF-κB binding sites with transcriptionally inactive homodimers. Furthermore, the p50 and p52 subunits can serve as a helper subunit by increasing the affinity of a transactivating subunit for a κB motif. The physiological role of p50 was directly assessed by gene disruption experiments. Mice lacking the p50 subunit show no developmental abnormalities, but display defects in the immune response. The expression of some NF-κB target genes is unimpaired in the p50 knock-out animal, suggesting that in some cases p50 can be functionally replaced by other subunits [9]. The targeted disruption of the transactivating relB subunit leads to a more dramatic phenotype including multiorgan inflammation and hematopoietic abnormalities [10].

2. Molecular biology of the inhibitory subunits

With the exception of very few cell types, including mature B cells, certain T cell lines, monocytes and neurons the DNA binding activity of NF-κB is not detected in unstimulated cells. Treatment of cytosolic fractions with dissociating agents generates the DNA-binding activity of NF-κB, by releasing an inhibiting factor, termed IκB [1,11]. Subsequent purification revealed the existence of 2 IκB variants: the IκB-α protein has a molecular weight of 37 kDa and IκB-β of 43 kDa. Cloning of IκB-α from chicken, pig, rat and human and of IκB-β from mouse revealed that these proteins also contain ANK repeats like the C-terminal portions of p105 and p100. NF-κB-inhibiting activity was also found for the 2 precursor molecules p105 and p100. Further members of the IκB family are the Bcl-3 protein and alternatively spliced forms of p105, called IκB-γ. The family of proteins belonging to the IκB family is now comprising 6 different proteins, which preferentially interact with different members of the NRD domain family. A structural characteristic of all IκB molecules is that they contain 5–7 complete ANK repeats. With the exception of Bcl-3, which is a predominantly nuclear protein, the IκB proteins retain their target molecules in the cytosol by masking their NLS sequence. The ability of IκB-α, IκB-β and p105 to dissociate a complex formed between NF-κB and its cognate DNA enables these proteins to attenuate ongoing transcription [12]. The domain responsible for the inhibition of DNA-binding has been assigned to a PEST-like sequence in the C-terminus of IκB-α.

3. Activation of NF-κB by pathogens and toxins

3.1. The role of reactive oxygen intermediates

Numerous, seemingly unrelated, stimuli lead to the activation of NF-κB. The physiological inducers of NF-κB all have in common that they are either primary pathogenic conditions that menace the cell or are secondary signals, which are endogenously produced in response to pathogens. The signals include very diverse stimuli such as bacterial products (e.g. bacterial lipopolysaccharide (LPS)), viruses and their products, eucaryotic parasites, inflammatory cytokines (e.g. tumor necrosis factor (TNF) and interleukin 1 (IL-1)), T cell mitogens and apoptotic and necrotic stimuli such as UV light, oxidants and γ-ray exposure [2]. Furthermore the DNA-binding form of NF-κB can be induced by the treatment of cells with toxins such as asbestos or some metals such as nickel. All these different inducers of NF-κB activity use different receptor systems and intracellular signal-transducing pathways but they ultimately cause the same reaction in the cytosol, which is the release of IκB from NF-κB.

Apparently most if not all of the diverse inducers of NF-κB activity have in common that they lead to the generation of reactive oxygen intermediates (ROIs), the phosphorylation of IκB and finally to its proteolytic degradation. The first indications for the probable involvement of redox signals came from the observation that the antioxidative compound *N*-acetyl-L-cysteine (NAC) can suppress the activity of NF-κB [13]. Also other antioxidants, such as 2-mercaptoethanol, dithiocarbamates, α-lipoic acid, butylated hydroxyanisol and chelators of iron and copper ions were found to prevent or impair the generation of active NF-κB. The chemical diversity of these compounds suggests that not their particular structure but their common antioxidative potential is responsible for their inhibitory effect. Overexpression of the antioxidative enzymes thioredoxin and catalase suppress NF-κB activation. The HIV-1 Tat protein, which is a strong inductor of NF-κB, was found to suppress the expression of Mn-dependent superoxide dismutase, a mitochondrial enzyme that is part of the cellular defense system against oxidative stress. Direct evidence for the activating role of ROIs comes from the inducing effect of micromolar amounts of hydrogen peroxide and mitomycin C in HeLa and Jurkat cells [14]. Reagents leading to the generation of superoxide, nitrous oxide, singlet oxygen and hypochlorite fail to activate, suggesting that NF-κB is a peroxide-inducible transcription factor, similar to the bacterial oxyR protein. Many of the different inducers of NF-κB were shown to increase the cellular production of hydrogen peroxide. These include TNF, IL-1, LPS, UV-A, γ-rays and okadaic acid, as measured by electron spin resonance techniques, the determination of lipid peroxidation products, the depletion of glutathione levels or chemoluminescent methods. The generation of hydrogen peroxide (or secondary ROIs) is common to most (if not all) inducers of NF-κB, because the inducing effects of TNF, IL-1, phorbol ester, 5 viral transactivator proteins, muramyl peptides, LPS, γ-rays, UV-A, leukotriene B4 and double-stranded RNA could be efficiently blocked by antioxidative compounds. The fact that all these different inducers are inhibited by antioxidants indicates that the generation of hydrogen peroxide may be a relatively late event in the signalling cascade activating NF-κB. It is currently not clear by which enzymes the ROIs are produced. Candidates are plasma membrane-associated NADPH-oxidases, xanthine oxidase, glucose oxidase and enzymes involved in arachidonic acid metabolism. In the case of TNF it was shown that the NF-κB-activating and cytotoxic effect relies on the production of ROIs generated in the mitochondria [15]. The electron transport inhibitors amytal and rotenone prevented NF-κB activation and blocked cell killing by TNF. Cells depleted of mitochondria by treatment with ethidium bromide were found to be insensitive to the cytotoxic and NF-κB-activating effect of TNF.

How can hydrogen peroxide activate NF-κB? Various kinases and phosphatases are known to be regulated in their activity by the redox status of the cell but the next known step in the

activation cascade of NF-κB is the phosphorylation of IκB.

3.2. The role of kinases and phosphatases

Direct phosphorylation of IκB is evident from the induction of a more slowly migrating IκB variant in SDS-gels in response to stimulation of cells with PMA, TNF, IL-1 and LPS. Treatment of extracts with phosphatases converts the faster migrating form of IκB into the more slowly migrating form. Stimulation of HeLa cells which are metabolically labelled with ^{32}P and subsequent immunoprecipitation with α-IκB antibodies revealed an increase in the phosphorylation of the constitutively phosphorylated IκB-α molecule. The analysis of IκB point mutants identified the serine residues at positions 32 and 36 to be the the targets for the inducible phosphorylation [16–18]. These 2 serines can be potentially phosphorylated by casein kinase II, but the kinase (or phosphatase) directly acting on IκB still awaits its identification. There are several candidate kinases that may be involved in the activation of NF-κB. UV-induced transcriptional activation of the NF-κB-dependent HIV-LTR was inhibited by overexpressed dominant negative forms of v-*src*, Ha-*ras* and raf-1 kinase and activated by v-*src*. Accordingly, tyrosine kinase inhibitors such as AG213, herbimycin A and tyrphostin 47 impaired the inducibility of NF-κB. The importance of ha-Ras and Raf-1 for NF-κB activation is supported by several other reports. Biochemical studies identified a ζPKC-associated kinase to be an important member of the signalling cascade leading to the phosphorylation of IκB-α. This IκB-kinase was suggested to have a molecular weight of approximately 50 kDa as predicted by in-gel-kinase assays. The analysis of transcriptional effects of transdominant mutants suggests a stimulating role of the double-stranded RNA-dependent protein kinase on κB-dependent gene expression. However, this kinase is only required for activation of NF-κB by double stranded RNA, while activation by TNF-α does not. The activation of NF-κB by anti CD28 antibodies involves the rapamycin-sensitive p70 S6 kinase pathway. But also phosphatases may be of importance for NF-κB activation, because a constitutively active form of the phosphatase calcineurin activates NF-κB in synergy with phorbol ester by inactivating IκB.

As seen in co-immunoprecipitation assays the newly phosphorylated form of IκB is found still attached to both DNA-binding subunits. The phosphorylation of IκB tags it for its proteolytic degradation. The involvement of the proteasome in the degradation of IκB is evident from studies employing specific membrane-permeable peptide inhibitors of the chymotrypsin-like subunit of the proteasome, which can significantly increase the half-life of IκB-α after stimulation of cells [7,19]. The different signal transduction pathways leading to the activation of NF-κB are schematically displayed in Fig. 1.

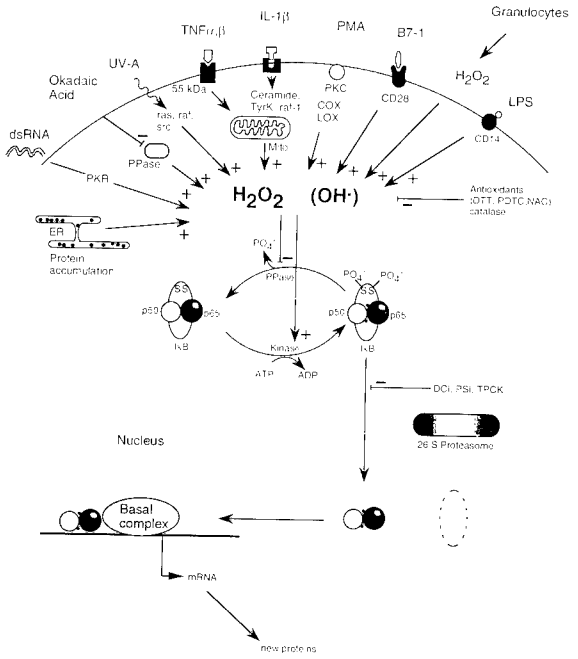


Fig. 1. Schematic representation of activation pathways for NF-κB. B7-1, B-cell activation antigen 7-1; COX, cyclooxygenase; DCI, 3,4 dichloroisocoumarin; dsRNA, double stranded RNA; DTT, dithiothreitol; ER, endoplasmatic reticulum; 55kDa, 55 kDa TNF receptor; LOX, lipoxygenase; Mito, mitochondrion; PDTC, pyrrolidinedithiocarbamate; PKR, double-stranded RNA-dependent protein kinase; PPase, phosphatase; PSI, Cbz-Ile-Glu(o-t-Bu)-Ala-Leucinal; S, serines 32 and 36; TPCK, tosylphenylchloromethylketone; TyrK, tyrosine kinase. For further details, see text.

4. Conclusions

The identification and detailed characterization of the various steps of NF-κB activation will allow the development of new drugs that interfere with the transcription factor activities. Such drugs will find application for treatment of fatal acute syndromes involving abberant expression of inflammatory cytokines, such as toxic/septic shock, systemic inflammatory response syndrome (SIRS) and acute respiratory distress syndrome (ARDS). They may also be beneficial in the treatment of chronic inflammatory diseases including Crohn's disease, rheumatoid arthritis and multiple sclerosis.

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Toxicology Letters 82/83 (1995) 413–417

Toxicology Letters

Effects of oxidative stress induced by redox-enzyme modulation on rat hepatocarcinogenesis

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Abstract

Inducibility of oxidative stress by menadione-associated redox cycling activation under redox-enzyme modulated conditions was examined in F344 male rat liver, by monitoring 8-hydroxydeoxyguanosine (8-OHdG) levels in DNA and hepatocyte injury. Further, the treatment-associated liver tumor-initiating, -promoting and -progressing potentials were assessed in terms of development of enzyme-altered preneoplastic foci, neoplastic nodules and hepatocellular carcinomas. With or without menadione, redox-enzyme modulation consisting of increased cytochrome P450 reductase by phenobarbital (PB), depletion of glutathione by phorone, inhibition of DT-diaphorase by dicumarol, with or without further supplement of iron, caused both 8-OHdG production and hepatocyte necrosis. Thus-induced oxidative stress exerted liver tumor promoting-activity in *N*-nitrosodiethylamine (DENA)-initiated rats, but neither initiating activity when promoted by 0.05% PB diet for 64 weeks, nor progressing activity when the oxidative stress was given for 33 weeks to preneoplastic nodule-bearing rats which was induced by DENA.

Keywords: Oxidative stress; 8-Hydroxydeoxyguanosine; Menadione; Redox enzyme; Hepatocarcinogenesis; Initiation; Promotion; Progression; Rat

1. Introduction

Recently, attention has been focused on the issues of possible involvement of reactive oxygen species (ROS), particularly that produced endogenously, in the carcinogenesis mechanisms [1–4]. ROS can be endogenously produced during inflammation as well as aerobic metabolism of either exogenous or endogenous substances [5,6]. An involvement of ROS has been postulated in carcinogenesis caused by so-called non-genotoxic carcinogens such as $KBrO_3$, ferric nitrilotriacetate, peroxisome proliferators, choline-deficient diet and Aroclor + iron-dextran, as well as in mouse skin tumor promotion by phorbol esters [1–6]. Further, H_2O_2 and benzoylperoxide have been reported to possess tumor promotion and progression but not initiation potentials in mouse skin [1–6]. Moreover, a malignant transformation of cultured fibroblasts by activated neutrophils has been reported [1–6].

Quinones are known to produce ROS during their metabolism through 1-electron reduction by flavoenzymes such as NADPH-cytochrome P450 reductase, where quinones are reduced to semiquinone radicals, which in turn reduce dioxygen

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to superoxide anion, regenerating quinones and thus activiating redox cycling [7,8]. The superoxide anion produced undergoes dismutation into H_2O_2, and in the presence of certain metal ions such as iron, H_2O_2 and superoxide anion can react to form more deleterious ROS, including hydroxyl radicals. Quinones also undergo direct 2-electron reduction by DT-diaphorase without producing ROS. An endogenous quinone, menadione, can induce hepatocyte injury via oxidative stress in either isolated or cultured rat hepatocytes [9].

In the present study, we developed an oxidative stress-inducing system in rat liver in vivo by adopting the menadione-associated redox cycling activation under redox-enzyme modulation that included increased cytochrome P450 reductase by phenobarbital Na (PB-Na), depletion of glutathione by phorone, inhibition of DT-diaphorase by dicumarol and supplementation of iron by EDTA (Fe) [10,11]. Further, using this system, tumor initiation, promotion and progression potentials of endogenously induced oxidative stress were examined in rat liver.

2. Materials and methods

2.1. Animals and diets

Fischer 344 male rats, 6 weeks old, (Nihon SLC Co. Ltd, Shizuoka, Japan) were used in all the experiments. Each experimental group contained 5–10 animals. The animals were maintained on a commercial stock diet, Oriental MF (Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum.

2.2. Experimental protocols

2.2.1. Redox-enzyme modulation treatment

Animals received daily i.p. injections of PB-Na (100 mg/kg body wt.) for 5 days, then received a single i.p. injection of phorone (200 mg/kg body wt.), EDTA (Fe) (70 mg/kg body wt.), dicumarol (25 mg/kg body wt.) and then a single i.g. intubation of menadione (50 mg/kg body wt.), respectively, at 80, 100 and 120 min after the phorone administration. For the vehicle treatment, each chemical was substituted by, respectively, saline, corn oil, saline, 0.825 M Tris–HCl (pH 9.7) and olive oil.

2.2.2. Induction of 8-hydroxydeoxyguanosine (8-OHdG) and hepatocyte injury

Animals received the redox-enzyme modulation treatment as mentioned above twice on days 6 and 7 after the daily injection of PB-Na for 5 days, and then were sacrificed 4 h after the final treatment. Hepatocyte injury was assessed by serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and histological examination. 8-OHdG in liver DNA was measured using HPLC connected with EC-detector as previously described [10].

2.2.3. Initiation potential

Animals received redox-enzyme modulation twice on days 6 and 7 after PB-Na injections as mentioned above, followed by 2/3 partial hepatectomy (PH) 4 h after the last treatment. After a 2-week recovery period, animals received promotion by 0.05% PB diet feeding for 64 weeks and were sacrificed. Livers were examined histochemically, immunohistochemically and histologically on the development of γ-glutamyltransferase (GGT)- and glutathione *S*-transferase placental form (GST-P)-positive preneoplastic foci and neoplastic nodules.

2.2.4. Promotion potential

Animals received initiation by *N*-nitrosodiethylamine (DENA; 200 mg/kg body wt., i.p.) followed by either redox-enzyme modulation treatment every other week as mentioned above except daily injection of PB-Na for 3 days, or 0.05% PB diet feeding, for 33 weeks, and were sacrificed. Livers were examined on the development of GGT-positive foci and neoplastic nodules.

2.2.5. Progression potential

Preneoplastic liver nodule-bearing rats which were induced by DENA-initiation followed by a selection by Cayama-Farber procedure [11], re-

ceived either redox-enzyme modulation every other week as mentioned above, or 0.05% PB diet feeding, for 33 weeks, and were sacrificed. Livers were histologically examined on the development of neoplastic nodules and hepatocellular carcinomas. Further, the preneoplastic nodule-bearing rats were given a single treatment of redox-enzyme modulation, and were sacrificed 24 h later. Livers were examined for hepatocyte injury.

3. Results

3.1. Induction of 8-OHdG and hepatocyte injury

Results are shown in Fig. 1. Menadione-associated redox cycling activation under redox-enzyme modulated condition by PB-Na + phorone + dicumarol with or without EDTA (Fe), exerted oxidative DNA damage, 8-OHdG production, as well as hepatocyte injury which was reflected in the elevated serum AST and ALT levels. Histologically, relatively large masses of necrotic hepatocytes were seen in zones 2 and 3, and on the liver surface. Treatment of each chemical alone exerted neither significant 8-OHdG production nor hepatocyte injury. Furthermore, PB-Na + phorone + dicumarol treatment without menadione, unexpectedly exerted significant 8-OHdG production and hepatocyte injury.

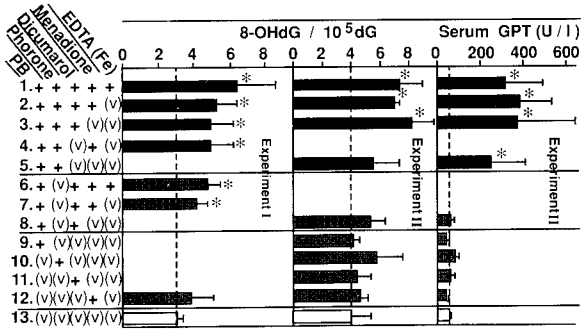


Fig. 1. Induction of 8-OHdG and hepatocyte injury by redox-enzyme modulation in rat liver. *Significantly different from group 13 ($P < 0.05$–0.001).

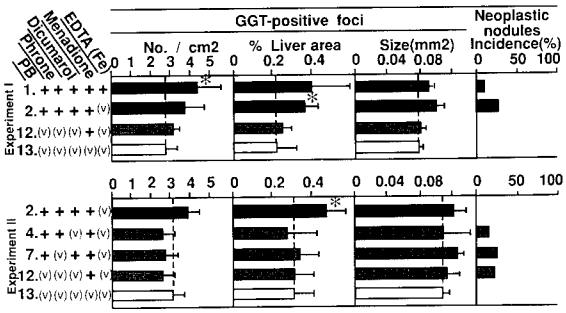


Fig. 2. Effects of redox-enzyme modulation on the induction of GGT-positive preneoplastic foci and neoplastic nodules in rat liver promoted by 0.05% PB diet for 64 weeks. *Significantly different from group 13 ($P < 0.05$).

3.2. Initiation potential

Results are shown in Fig. 2. When promoted by PB diet, menadione-associated redox cycling activation under redox-enzyme modulation by PB-Na + phorone + dicumarol with or without EDTA (Fe) appeared to slightly exert GGT-positive foci development, since either the number of foci or percentage liver area occupied by foci was slightly but significantly increased compared with the vehicle-alone group. The treatment, however, exerted significant increased development of neither neoplastic nodules nor GST-P-positive foci (data not shown). Thus, the treatment-associated oxidative stress exerted no clear tumor initiation potential under PB diet promotion.

3.3. Promotion potential

Results are shown in Figs. 3 and 4. Menadione-associated redox cycling activation under redox-enzyme modulation by PB-Na + phorone + dicumarol + EDTA (Fe) exerted tumor promotion potential, with both GGT-positive foci and neoplastic nodule developments being significantly increased compared with vehicle-alone as well as PB-Na-alone groups.

3.4. Progression potential

Results are shown in Fig. 5. Menadione-associated redox cycling activation under redox-en-

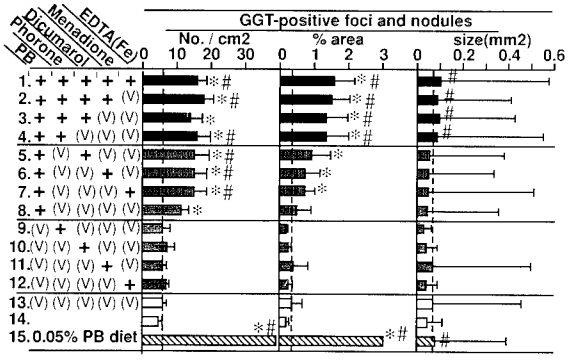


Fig. 3. Promotion effects of redox-enzyme modulation on the induction of GGT-positive preneoplastic foci and nodules in rat liver initiated with DENA. Significantly different from group 13 (*) and from group 8 (#) ($P < 0.05$–0.001).

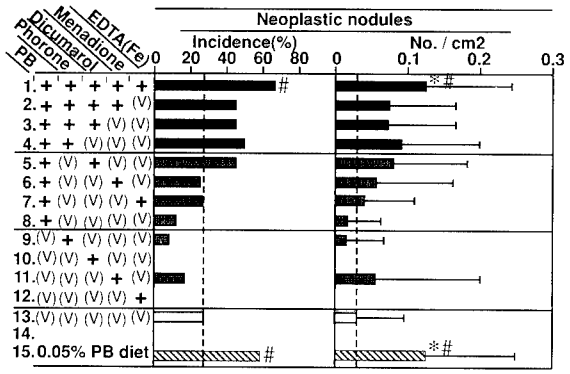


Fig. 4. Promotion effects of redox-enzyme modulation on the induction of neoplastic nodules in rat liver initiated with DENA. Significantly different from group 13 (*) and from group 8 (#) ($P < 0.05$).

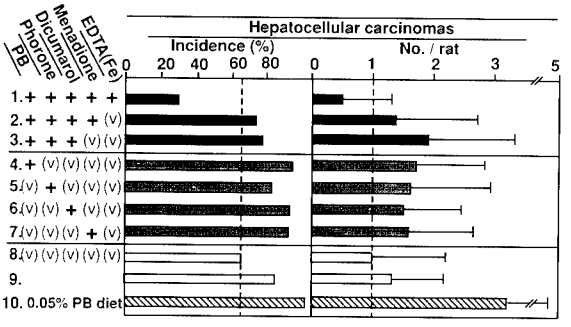


Fig. 5. Effects of redox-enzyme modulation on the evolution of preneoplastic nodules into hepatocellular carcinomas in rat liver. The number per rat in group 1 was significantly decreased compared with group 9 ($P < 0.05$) but not with group 8.

zyme modulation of PB-Na + phorone + dicumarol with EDTA (Fe) exerted no progression potential but rather inhibitory effect on the evolution of preneoplastic liver nodules into hepatocellular carcinomas and neoplastic nodules, where number of neoplastic nodules per rat, particularly that larger than 5 mm in diameter, 4.6 ± 2.1 were significantly decreased compared with 7.5 ± 3.3 in vehicle-alone group ($P < 0.05$). Further, the preneoplastic nodules histologically exhibited rather resistance to the induction of the treatment-associated hepatocyte injury.

4. Discussion

In the present study, it was indicated that oxidative stress can be induced endogenously by menadione-associated redox cycling activation under redox-enzyme modulation in rat liver in vivo. Further, thus-induced oxidative DNA damage was not associated with any clear liver tumor-initiating activity, at least that could be promoted by PB. In contrast, thus-induced oxidative stress exerted liver tumor-promoting activity, suggesting that compensatory regeneration following the oxidative tissue injury can be a tumor-promoting stimulus [12]. In this context, it would be worth noting a report made by Tennant et al. [13] that toxic injury is not always associated with induction of chemical carcinogenesis. On the other hand, thus-induced oxidative stress exerted no liver tumor progression potential, probably partly due to resistance of the preneoplastic liver nodules induced by the Cayama-Farber procedure, against the induction of oxidative stress caused by the present treatment, suggesting that progressing potential of oxidative stress, if any, might depend upon not only the oxidative stress-inducing procedure but also the natural history of the nodule development during which the nodules should have acquired either resistance or adaptation.

In conclusion, it was suggested that oxidative stress induced endogenously by redox-enzyme modulation can be a tumor-promoting stimulus.

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Toxicology Letters

Antisense phosphorothioate oligodeoxynucleotides: introductory concepts and possible molecular mechanisms of toxicity

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Abstract

Over the past 5 years or so, much attention has been given to the possible use of synthetic antisense oligonucleotide analogs as a new class of therapeutic agents that function by sequence-specific inhibition of genetic expression. The basic design concepts which underline this novel approach to drug discovery are briefly described herein, together with some of the chemical, biochemical, and pharmacological aspects of phosphorothioate oligodeoxynucleotides that are first-generation antisense compounds now under clinical investigation. Possible molecular mechanisms of toxicity for this class, and other structural types of antisense compounds are discussed with the hope of stimulating interest in future toxicological studies in this emerging area of drug development.

Keywords: Antisense; Phosphorothioate; Oligodeoxynucleotides; Inhibitors of genetic expression; Molecular mechanisms of toxicity

1. Introduction

Research employing antisense oligonucleotides has rapidly led to a number of reviews and comprehensive edited books on this subject [1–4], which should be consulted to obtain a complete picture of the current scope and directions of this new investigative field. What follows is a very brief introductory overview and then short synopses of some of the current clinical trials and possible molecular mechanisms of toxicity that warrant attention.

Drug design through control of genetic expression by means of an 'antisense oligonucleotide' refers to use of synthetic oligonucleotides or functional analogs thereof to bind in a sequence-specific manner to preselected RNA 'targets' and thus block translation of the corresponding protein. The underlying physicochemical principle for this interception process is hydrogen bonding of the type first described in the 1950s by Watson and Crick for A·T and G·C basepairs in complementary nucleic acid sequences with antiparallel orientation (Fig. 1). The 'sense' or coding strand of a gene by definition gives rise to the complementary 'sense' RNA sequence that is in turn translated into the protein encoded by that particular gene. Consequently, interception of sense RNA requires use of the complementary, or by definition, 'antisense' oligonucleotide sequence. These sense and antisense relationships are depicted in Fig. 1 for interception of

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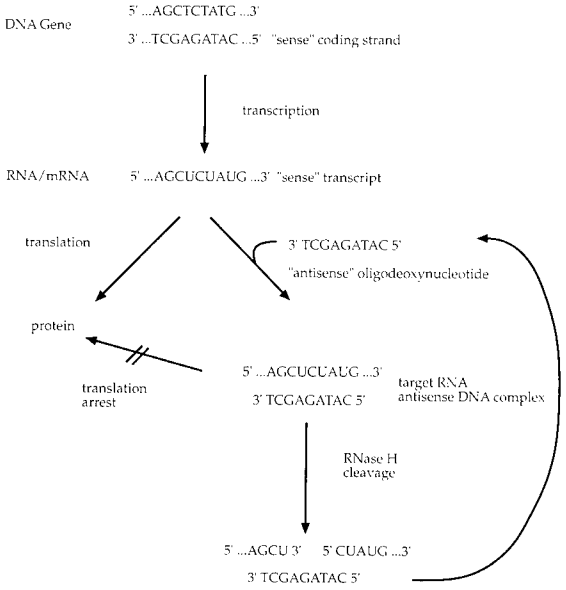


Fig. 1. Schematic representation of the mechanism of action for sequence-specific inhibition of genetic expression by use of an 'antisense' oligodeoxynucleotide to hybridize to the reverse complementary sequence of mRNA encoded by the 'sense' strand of DNA, and thus form a complex which prevents translation of a pre-selected protein and/or leads to cleavage of the mRNA strand by RNase H.

sense RNA by an oligodeoxynucleotide antisense agent. A conceptually similar but technically different approach uses vector-encoded, endogenously expressed antisense RNA transcripts. A deoxy or DNA-like antisense oligonucleotide offers the potential advantage of cleavage of the sense RNA target by RNase H and, hence, theoretically more effective inhibition than that achievable by either endogenously expressed RNA or an exogenous synthetic RNA-like oligomer, both of which can form a complex with target RNA but are not cut by RNase H. In this regard, it is worth noting that only certain structurally modified oligodeoxynucleotide analogs, as mentioned below, retain sufficient resemblance to DNA to allow formation of a complex with target RNA that is a substrate for RNase H.

In principle, this interception process may involve targeting either unspliced RNA in the nucleus or spliced mRNA in the cytoplasm. Although any RNA or mRNA sequence can in theory be targeted, it is expected a priori (and found experimentally) that only certain target RNA sequences allow for successful interception by the antisense oligonucleotide. RNAs have complex secondary and tertiary structures that make many sequences unavailable for binding owing to their involvement, through hydrogen bonding, in either basepaired 'stem' structures or some other intramolecular structural element. Additional interference with target interception presumably arises from proteins that are bound to RNA and thus prevent its coming into contact with an antisense agent.

2. Hybridization to target RNA and cross-hybridization to non-target RNA

The binding of antisense oligonucleotide to RNA that is achievable theoretically is dictated by the laws of thermodynamics for molecular systems under equilibrium conditions, such as those which are operative during the use of synthetic oligodeoxynucleotides as hybridization probes, sequencing primers, or PCR primers, which are now commonly employed techniques. The specificity of antisense oligonucleotide hybridization with target RNA versus one or more non-target RNAs is related to the efficacy and, possibly, the toxicity of the antisense agent to the extent that the former event triggers the intended inhibition of expression while the latter event(s) block expression of non-target gene products. While the consequences of this blocking of non-target protein synthesis are unpredictable, statistics and the physical chemistry of nucleic acids allow one to make the following general (albeit oversimplified) assessments.

An approximately 13-mer antisense oligonucleotide has, from a statistical viewpoint, adequate sequence 'information' to uniquely bind to a given target RNA in a 'background' of all other expressed RNAs, although most studies generally employ 18- to 21-mers by analogy to what is commonly used for probes and primers. Although these and even longer oligonucleotides offer statistically greater probability of unique hybridization to the particular target RNA, as

well as tighter binding that could lead to increased efficacy, thermodynamic equilibrium may actually not be achievable in cells, wherein the kinetics of binding may instead dominate. Hybridization conditions for use of probes and primers can generally be optimized by adjusting temperature, salt concentration, and the ratio of probe or primer to target. By contrast, the conditional specificity for hybridization of antisense oligonucleotides in cells can only be controlled by decreasing the concentration of the antisense agent and/or shortening its chainlength to decrease the binding affinity and thereby giving up, statistically, target-sequence uniqueness relative to 'background' RNAs. Even with shortening of the antisense oligonucleotide there will most likely be some degree of cross-hybridization to non-target RNAs that have partially complementary sequences characterized by 1, 2, etc. mismatched basepairs. These partially complementary complexes with non-target RNA are less stable than the fully complementary complex with target RNA, but they may nevertheless form under physiological conditions, especially at high concentrations of antisense oligonucleotide. Consequently, all antisense oligonucleotides can be expected to show more of the resultant hybridization-based toxicities as concentrations of the antisense agent are increased.

Because of these complexities in biochemically dynamic living cells, and in view of the above mentioned inability to reliably predict which RNA sequences will be both accessible and mechanistically productive when complexed with an intended antisense agent, the design and use of antisense oligonucleotides is largely an empirical process with few if any proven rules at this time. On the other hand, most antisense reports have used 15- to 24-mers targeted to the translation start site that is assumed to be relatively open to hybridization. This empiricism also applies to sequence changes to reduce unwanted toxicities that derive from hybridization to non-target RNAs. Consequently, unlike small molecules, the antisense drug design approach easily allows one to empirically change sequence in an attempt to maintain efficacy but eliminate hybridization-based toxicity.

3. Properties of antisense analogs and efficacy of phosphorothioates

A presumptive cardinal feature of any antisense drug is that it has adequate resistance to degradation by exo- and endonucleases that are ubiquitous in serum and cells. This was recognized by Zamecnik and Stephenson in their seminal studies [5] with Rous sarcoma virus on the antisense approach which investigated protection of an antiviral oligodeoxynucleotide by use of 3′,5′-terminal modification as an isourea derivative. There are now available many types of 3′ and 5′ modifications reported to afford some degree of protection against degradation of native phosphodiester linkages (Fig. 2, $X = O^-$) by 3′ (primarily) and 5′ exonucleases. However, recent evidence [6] for endonuclease activity suggests that more extensive, if not complete, modification of an antisense oligonucleotide analog may be required. Fully-modified antisense agents were originally studied by Jayaraman et al. [7], who pioneered the use of oligodeoxynucleotide methylphosphonate analogs (Fig. 2, $X = CH_3$), which also allowed investigation of nonionic, relatively hydrophobic, antisense agents that were envisaged by these researchers as having the potential for passive uptake into cells by penetrating the lipid bilayer

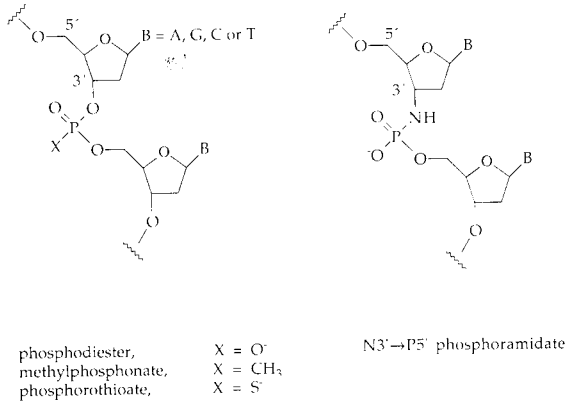


Fig. 2. Schematic structures of the generalized repeating unit of oligodeoxynucleotide with either phosphodiester ($X = O^-$), methylphosphonate ($X = CH_3$), phosphorothioate ($X = S^-$), or phosphoramidate linkages.

of outer membranes. However, recent results indicate that this potential advantage and uptake mechanism are not operative [8].

Shortly following these early studies by Zamecnik and Stephenson and Jayaraman et al., several reports [9–11] appeared on the use of phosphorothioate oligodeoxynucleotide analogs (Fig. 2, X = S^-). Subsequently, a large number of other types of possible antisense structures have been investigated. 2′-Deoxy- and ribooligonucleotide analogs with either phosphate modifications or non-phosphate linkages, 2′-*O*-alkyl analogs, α-anomers and many more have been reviewed elsewhere [1–4]. A very promising class of second-generation antisense agents are the N3′ → P5′ phosphoramidates (Fig. 2) reported by Gryaznov et al. [12].

That there is significant uptake of antisense phosphorothioate oligodeoxynucleotides by cells was initially viewed by many with surprise; however, it is now widely accepted that, based on observed efficacy versus sequence-controls, functionally adequate cellular uptake does take place, at least in those particular systems. What is not currently understood are the mechanistic details for either the internalization or intracellular trafficking processes. Oligonucleotide uptake and distribution are well beyond the scope of the present overview, and have been recently reviewed [13–15].

Also available are recent reviews [16,17] that compile references to over 60 publications through 1994 focusing on the control of genetic expression in cell culture using antisense phosphorothioate oligonucleotides, many of which are related to drug discovery. One of these reviews [17] also provides references to over 20 reports through 1994 of in vivo studies of intended antisense phosphorothioate agents in mice, rats, and pigs, as well as chick embryos, ducks, and even goldfish! To complement this promising in vivo information, there are also recent reviews [18,19] and up-to-date reports [20,21] on the pharmacokinetics and primary metabolism (i.e. nuclease-mediated degradation) of phosphorothioate oligodeoxynucleotides. The current consensus for the pharmacokinetics of phosphorothioate oligodeoxynucleotides involves biphasic clearance from plasma ($t^{\alpha}_{1/2} \sim 10$ min and $t^{\beta}_{1/2} \sim 24$ h) with slow elimination in urine ($t_{1/2} \sim 24$ h).

4. Human clinical trials with antisense phosphorothioate oligodeoxynucleotides

All of the current clinical trials with antisense agents as investigational new drugs are being conducted with fully phosphorothioated oligodeoxynucleotides and are listed in Table 1. Although it is too early to draw conclusions regarding efficacy of these investigational new drugs for treatment of the indicated diseases, unpublished results (P. Iversen, personal communication) from a study involving continuous i.v. infusion have shown that administration of a phosphorothioate analog targeted to *p53* mRNA at doses up to 0.25 mg/kg/h for 10 days is well-tolerated

Table 1
Human clinical trials with antisense phosphorothioates

Disease	Gene target	Status	Investigator(s)
Acute myelogenous leukemia (systemic infusion)	p53	Phase I	University of Nebraska Medical Center/Lynx
Acute myelogenous leukemia (marrow purge)	p53	Phase	University of Nebraska Medical Center/Lynx
Chronic myelogenous leukemia (marrow purge)	c-*myb*	Phase I	University of Pennsylvania Cancer Center/Lynx
Chronic myelogenous leukemia (systemic infusion)	c-*myb*	Phase I	University of Pennsylvania Cancer Center/Lynx
Genital warts (local application)	HPV	Phase II	Isis
AIDS-related retinitis (intraocular)	CMV	Phase I	Isis
AIDS (subcutaneous infusion)	HIV	Phase I	Hybridon
Restenosis following balloon angioplasty	c-*myc*	Phase I	Various investigators in Argentina/Lynx

by patients with either acute myelogenous leukemia or myelodysplastic syndrome. For a 75-kg patient, this infusion rate translates to 4.5 g of the investigational antisense agent, much of which is slowly excreted and low-levels (~0.5 μM) of which are maintained in blood plasma.

5. Possible molecular mechanisms of toxicity of antisense oligonucleotide analogs

Already mentioned is hybridization of the antisense oligonucleotide analog to a fully or partially complementary sequence present in non-target RNA. This is obviously a sequence-dependent process and, hence, in principle is avoidable by changing sequence to maintain efficacy but lessen toxicity.

Similar comments apply to inadvertent hybridization of the antisense oligonucleotide analog to DNA by duplex (or triplex [1]) formation, which blocks either expression or binding of a transcription factor.

Much more problematic is toxicity arising from sequence-independent binding of the antisense oligonucleotide analog to key structural proteins, enzymes, receptors, or growth factors via bonding (ionic or hydrophobic) to the modified backbone of the antisense oligonucleotide analog, inasmuch as this is an inherent property of the antisense agent. In this instance the resultant toxicity is not avoidable by changing sequence and therefore would necessitate a change in backbone or use in an alternative clinical indication, or an alternative mode of delivery, or use of compensatory drug treatment. An example of a toxicity that is avoided by use of an alternative mode of delivery is hypotension reported [22] for rapid (bolus) i.v. administration of phosphorothioate oligodeoxynucleotides that is completely avoidable by use of slow (continuous) i.v. administration. Another example is chelation of metal ions by phosphorothioate oligodeoxynucleotides (P. Iversen, personal communication), which might be ameliorated by supplementation through co-infusion of appropriate salts.

Another possible toxicity may derive from nuclease degradation to nucleotide 5′ thiophosphates that can, in principle, be converted to 5′ triphosphates and thus lead to de novo biosynthesis of genomic DNA having a low level of potentially mutagenic phosphorothioate linkages. Alternatively, the nucleotide 5′ thiophosphates may function as antimetabolites that produce toxic side-effects.

Clearly, there are many novel and interesting toxicology issues to investigate, as well as regulate from an FDA perspective [23]. The challenges to do so are being addressed, and will undoubtedly stimulate further successful efforts in this emerging area of drug development.

Acknowledgements

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Pharmacology and toxicology of phosphorothioate oligonucleotides in the mouse, rat, monkey and man

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Abstract

Phosphorothioate oligonucleotides (PS-ODN) designed to temporarily modulate selected gene expression have made the journey from bench top to bedside in a remarkably short period of time. A PS-ODN with sequence complementary to the p53 mRNA was administered to mice (4 mg/kg subcutaneously), rats (3–300 mg/kg intravenously), monkeys (intravenous infusions for up to 15 days) and humans (up to 0.25 mg/kg/h intravenous infusions for 10 days). These studies demonstrate the PS-ODN provides feasible pharmacokinetic parameters and minimal toxicity.

Keywords: Phosphorothioate oligonucleotides; Preclinical pharmacology; DNA damage; p53 mRNA; Gene-specific therapeutics

1. Introduction

Interruption of gene expression, either temporarily or permanently, for the purpose of modulating an unwanted cellular activity is the rationale for gene-specific therapeutics. An antisense approach in which a nuclease resistant, synthetic, single-stranded DNA with nucleic acid sequence complementary to a selected target mRNA is being viewed with increasing acceptance as a model of gene-specific therapeutics. Many reports in the literature demonstrate the utility of the antisense approach in cell culture and in vivo. However, studies of the potential toxicity of oligodeoxyribonucleotides are few and restricted to modest exposures in limited numbers of animal species. The purpose of this report is to describe the pharmacokinetic behavior and observed toxicity of a phosphorothioate oligonucleotide with sequence antisense to p53 mRNA.

New therapeutic agents for treatment of acute myelogenous leukemia (AML) must be identified that are capable of enhanced remission rates, diminished treatment-related mortality or that can achieve remissions in refractory patients. p53, a tumor suppressor protein, is a DNA-damage responsive protein. The expression of p53 protein is augmented in response to several DNA damaging agents including radiation, mitomycin C and hydrogen peroxide [1–7]. Agents that do not directly damage DNA, such as the antimetabolite cytosine arabinoside, do not induce p53 protein [2]. Halogenated pyrimi-

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dines, used as radiosensitizers to produce direct DNA damage in the presence of ionizing radiation, cause the induction of GADD45, a downstream effector of p53 [8]. Hence, p53 mediates important consequences of DNA damaging agents including arrest in the cell cycle at G1/S and/or programmed cell death [9–11] (see Fig. 1).

Cell cycle checkpoints are essential for maintaining genomic integrity [12,13]. At least some of these signal transduction pathways, which arrest cells in G1/S and G2/M phases of the cell cycle in response to DNA damage, allow repair of DNA [12,13], preventing the transfer of heritable mutations and disallowing the segregation of damaged chromosomes [12,13]. Recently, p53 has been determined to be in the G1 arrest pathway induced by DNA-damaging agents [2,3,14]. Cells lacking p53 or cells containing dominant negative forms of p53 do not arrest in G1 phase of the cell cycle in response to ionizing radiation [3]. In addition, restoration of wild-type p53 into p53-negative cell lines restores the G1 arrest, and the addition of dominant negative forms of p53 to cell lines containing wild-type p53 inhibits the G1 arrest [14]. Inhibition of p53 by antisense oligonucleotides (ODNs) can recover rat hepatocytes from UV-induced G1 arrest [15]. Recently, WAF-1 protein, which is induced by p53, has been tentatively determined to mediate the G1 arrest by blocking Cdk enzymes resulting in the inhibition of DNA synthesis [16]. Thus, p53 appears to play the role of checkpoint pathway protein in G1/S similar to that found in the RAD9 gene discovered in *Saccharomyces cerevisiae*, which induces a G2/M delay in response to ionizing radiation [12,17,18].

Since p53 is involved in the G1/S checkpoint

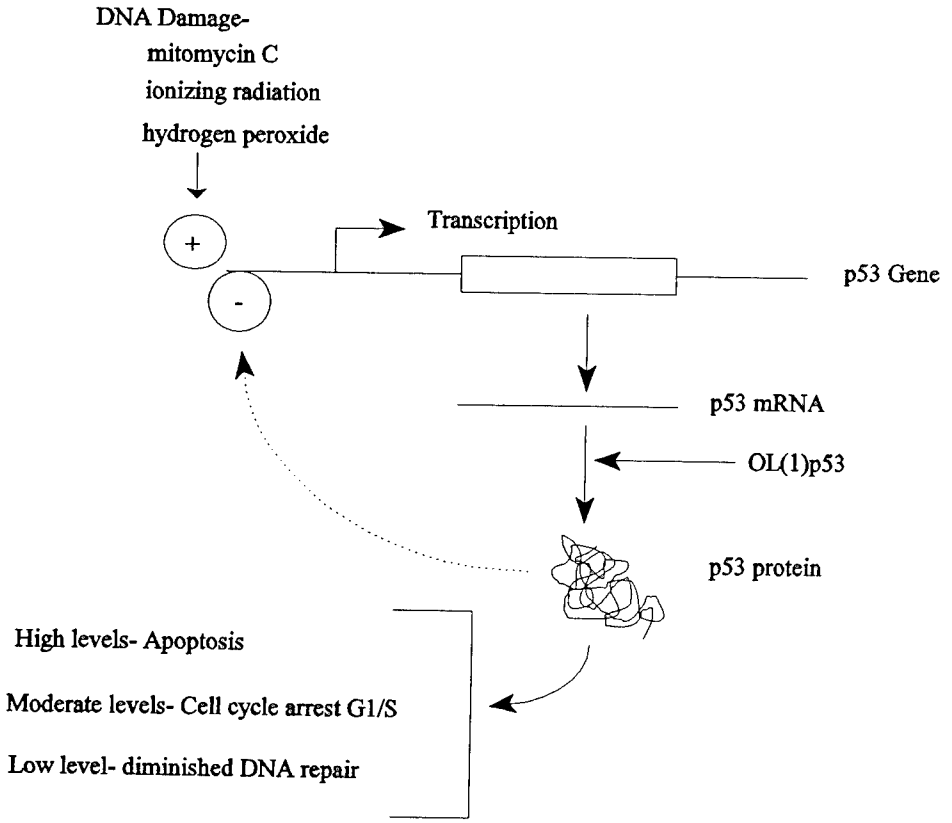


Fig. 1. Regulation of expression and phenotypes attributed to p53. The p53 promoter is influenced by DNA damaging agents which cause induction of p53 protein expression. The p53 protein acts as a negative regulator of the p53 promoter but the negative feedback may involve a cascade of interactions (dotted line). The OL(1)p53 oligonucleotide interferes with the process of translation which modulates the expression of p53.

pathway, loss of p53 may be expected to induce radiosensitivity or chemosensitivity similar to observations made in yeast lacking RAD9 protein [12,17,18]. Cells from patients with Ataxia-telangiectasia show no induction of p53 after exposure to ionizing radiation and, subsequently, no G1 arrest in the cell cycle [2]. These cells also exhibit radiosensitivity when exposed to ionizing radiation (for review see [19]). Mouse fibroblasts lacking p53 and normal human fibroblasts treated with an antisense ODN to p53 show increased chemosensitivity to direct DNA-damaging agents [20]. These results clearly show that lack of response by p53 or loss of p53 can induce radiosensitivity and chemosensitivity. Other groups, however, have shown that loss of p53 induces chemoresistance and radioresistance [21–24]. Thus, whether loss of p53 causes increased or decreased sensitivity to DNA-damaging agents may be cell-type specific, disease state specific, or specific to various stages of development.

We have shown previously that antisense ODNs directed against p53 cause a decrease in human acute myelogenous leukemia (AML) blast cell viability [25]. p53 is expressed at higher levels in AML blast stem cells than normal hematopoietic cells [26,27], and p53 expressed in AML blast stem cells is predominantly wild-type [28–30]. These observations lead to a therapeutic interest in an antisense approach to the clinical management of AML.

OL(1)p53, 5′-d(CCC TGC TCC CCC CTG GCT CC)-3′ complementary to exon 10 region of the human p53 mRNA (molecular weight 6625 g/mole) was supplied by Lynx Therapeutics (Foster City, CA) as a sterile, apyrogenic, dry powder in vials each containing 50 mg. A radioactive OL(1)p53 was prepared according to the method of Stein et al. [31].

2. Results

2.1. Mouse

Four mice were examined following a 4 mg/kg application of OL(1)p53 subcutaneously. Blood samples were recovered at 2, 4, 8, 24, 48 and 72 h post-infusion and radioactivity in each sample determined. The concentration of OL(1)p53 was calculated from the specific activity of the injected material and reported as μg/ml. The radioactive material measured may be in the form of fully intact oligonucleotide and/or degradation products.

The elimination half-life of the single compartment model was observed. The volume of distribution of 1.71 l/kg is equivalent to 42.75 ml in a 25 g mouse, suggesting that OL(1)p53 is sequestered in addition to being distributed throughout the body.

2.2. Rat

Studies involved 3 rats at each of 5 dose levels (3.0, 10.0, 27.5, 100.0, and 300 mg/kg), administered intravenously as a bolus injection to 4 animals per dose group. Blood samples were withdrawn from the jugular vein via a surgically implanted catheter. The OL(1)p53 was injected under anesthesia into the right femoral vein.

The elimination half-life, concentration in plasma at time 0 (Cp0), area under the plasma concentration vs. time curve (AUC), amount excreted in the urine, and excretion rates were increased as a result of increased dose. However, the volume of distribution V_d and renal clearance were not increased as the dose was increased.

The volume of distribution observed in the rat is substantially smaller than that observed in the mouse. This is possibly due to the route of administration and the fact that the OL(1)p53 is injected as a bolus injection in the rat but in the mouse the release of OL(1)p53 is relatively slow from the subcutaneous site. This suggests the rate of distribution from the blood into tissue is relatively slow in comparison to the rate of loss due to excretion.

2.3. Monkey

Four hour infusions of 7.5 mg/kg OL(1)p53 were injected intraarterially into three Rhesus monkeys. A biphasic elimination from plasma was observed with an elimination half-life of 18.1 h. The volume of distribution was 4 l/kg, again suggesting that OL(1)p53 is widely distributed in the body and may be sequestered at some sites.

The clearance rate is 25.2 ml/min, which is less than the glomerular filtration rate. The maximal plasma concentration achieved was 1.93 μg/ml, which was predominantly full length material when evaluated by gel electrophoresis. From 13% to 27% of the dose was excreted into urine in 6 days. The liver, kidney, heart, spleen and pancreas were the organs of greatest accumulation.

Three monkeys were administered continuous intravascular infusions with doses of 8.3 mg/kg per day over a period of 6 to 15 days. Steady-state plasma concentration was observed in 4 to 9 days, reaching concentrations of 1.5 to 5.6 μM. The volume of distribution is equal following continuous infusion or a single 4 h injection. The maximal plasma concentrations following the infusion reached nearly five times that following the single injection. This is expected, as a new steady-state plasma concentration should be achieved in 3 to 5 half-lives.

The plasma half-life following the single injections was significantly shorter than that observed following continuous infusions. This is consistent with the higher plasma concentration and the smaller clearance.

2.4. Human

OL(1)p53 was administered as a 10 day continuous intravenous infusion to 17 patients with either relapsed or refractory acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS). Analysis of blood and urine by high performance electrophoretic chromatography (HPEC) and high performance liquid chromatography (HPLC) followed by post-labeling at the 5′-end with [^{32}P]ATP and polynucleotide kinase revealed approximately 36% of the recovered material in urine and, greater than 90% of the material recovered in plasma retains the identical electrophoretic mobility as OL(1)p53 and approximately 53% of the recovered material in urine is equivalent to mononucleotide monophosphorothioate material. A single patient was administered uniformly ^{35}S-labeled OL(1)p53, and the analysis confirms approximately 90% agreement with chromatographic methods with greater than 90% total recovery of labeled material. The plasma concentration and area under the plasma concentration curve (AUC) were linearly proportional to the dose (mg/m^2) of OL(1)p53. The elimination half-life increased as a function of dose from 24.4 h at 0.05 mg/kg per h to 62.5 h at 0.25 mg/kg per h. The renal clearance did not increase with increasing dose, remaining at 14.4 ± 2.56 ml/min, hence the renal clearance accounts for a smaller percentage of total clearance with increasing dose from 54.3 ± 6.6 at 0.05 mg/kg per h to 15.8 ± 3.3 at 0.25 mg/kg per h (Fig. 2). This apparently saturable renal clearance pathway suggests that glomerular filtration either may be limited by the amount of free oligonucleotide or is not the major mechanism for renal clearance. Therefore, non-renal clearance increases with dose, and the volume of distribution is increased from 34.1 l at 0.05 mg/kg per h to 454.1 l at 0.25 mg/kg per h. The half-life changed with increased dose in the rat, but the volume of distribution did not. This may have been due to the differences in the injections, as the rat received a bolus injection and the humans received a continuous infusion.

OL(1)p53 reaches an apparent steady-state plasma concentration in less than 24 h. The expected time to steady state would be between 3 and 5 half-lives, which would be several days. The difference between the observed and ex-

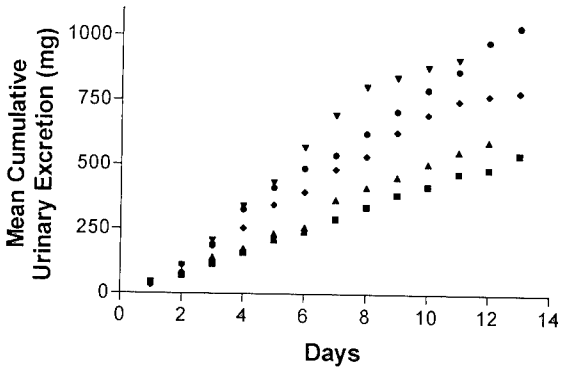


Fig. 2. Cumulative urinary clearance of OL(1)p53 in human patients. The appearance of OL(1)p53 in the urine was measured for each patient following a total of 5 different dosage regimens. Data are plotted as the average cumulative urinary excretion for all patients within each dose level of OL(1)p53. Dosages: ■, 0.05 mg/kg/h; ▲, 0.10 mg/kg/h; ▼, 0.15 mg/kg/h; ◆, 0.20 mg/kg/h; ●, 0.25 mg/kg/h.

pected time to reach steady state plasma concentrations is probably due to a high degree of plasma protein binding. We employed human serum albumin (HSA) protein bound to sepharose beads to determine the HSA-protein dissociation constant [32]. These observations indicate that OL(1)p53 is approximately 98% protein bound in plasma. Hence, the infusion establishes equilibration of plasma protein binding. The small fraction of free OL(1)p53 in blood plasma is then available for renal clearance, distribution throughout the body and uptake by target cells.

3. Conclusions

Once the promise of nuclease resistant phosphorothioate oligonucleotides has demonstrated in vivo efficacy [33–35] and feasible pharmacokinetic parameters [36–39], the task of identification of target cell sites and oligonucleotide functional bioavailability must be resolved. The most simple solution to this complex problem is to optimize the dose-scheduling of the oligonucleotide. The data presented here address the problem of matching the rate of target cell uptake with the rate of loss from systemic circulation. In this case analysis of OL(1)p53 pharmacokinetic behavior was established in the mouse, rat and monkey. These observations support the conclusion that target cell uptake is slow relative to the rate of elimination from plasma following a bolus injection. Hence, continuous exposure of cells to oligonucleotide will optimize target cell accumulation of OL(1)p53. It is not possible to extrapolate these observations to other target cells or for other oligonucleotide sequences or oligonucleotide chemistries. However, these observations should provide a basis for comparison.

Another substantial task is to elucidate the patterns and mechanistic causes of oligonucleotide toxicity in vivo. A detailed summary of the clinical toxicity is given in [40]. One patient developed transient, asymptomatic, nonoligouric renal failure which occurred after initiation of vancomycin and may have been the result of a drug interaction between the clearance of vancomycin and the OL(1)p53. We did not observe toxicity that could easily be linked to OL(1)p53 in either the preclinical pharmacology and toxicology studies or the phase I trial.

Acknowledgments

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Toxicology Letters 82/83 (1995) 431–434

Toxicology Letters

Novel enzymatic and immunological responses to oligonucleotides

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Abstract

Oligonucleotide phosphorothioates (PS-oligos) are being studied as antisense agents for viral infection and cancer. In preclinical studies, PS-oligos produced dose-dependent changes in heart rate and blood pressure and significantly reduced serum hemolytic complement, which could be avoided by slowing infusion rates. Here, in vitro PS-oligo treatment of either human, rhesus monkey or guinea pig serum reduced hemolytic complement and further inhibited in vitro coagulation when added to whole blood or citrated plasma. These effects were dependent upon both oligonucleotide dose and structure. Oligonucleotides having identical sequences but containing methylphosphonates (*Chimeric*), 2′-*O*-methyl ribonucleosides (*Hybrid*) or 3′ hairpin loop (*Self-stabilized*) had altered effects on complement and coagulation in vitro.

Keywords: Antisense; Complement; Coagulation; Oligonucleotides; Phosphorothioate

1. Introduction

Antisense oligonucleotides have been shown to inhibit gene expression in vitro and in vivo and are being explored as therapeutic agents [1]. Phosphorothioate-modified oligonucleotides (PS-oligos) show enhanced therapeutic potential as compared to 'natural' phosphodiester oligonucleotides [2,3]. Antisense PS-oligos are currently in Phase I/II trials for therapy of viral infections and cancer [4,5].

Preclinical studies of PS-oligos indicate that high-dose bolus i.v. infusions into non-human primates can be associated with acute hemostatic changes associated with measurable inhibition of circulating complement and/or coagulation activities determined by standard clinical assays [6–8]. Of note, these effects appear to be independent of nucleotide sequence, but dependent upon phosphorothioate modification, dose and infusion rate so that slow administration minimized observable side effects [6].

This study presents in vitro experimental models for studying the previously observed in vivo effects of PS-oligos on complement and coagulation. The PS-oligo studied here is GEM-91 [4,6,9], a 25-mer antisense oligodeoxynucleotide phosphorothioate, which is presently in

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clinical trials for HIV infection. Structurally-modified antisense oligonucleotide analogues of the PS-oligo were also studied for altered in vitro activities.

2. Results and discussion

Initial experiments used normal Rhesus monkey and human serum spiked in vitro with high-dose (250–500 μg/ml) PS-oligo [9] and assayed in parallel with serum spiked with control mouse genomic DNA. Results showed that both the classical and alternative pathways of complement measured by standard hemolytic assays [10] were reduced 25–60% by PS-oligo as compared to treatment with equivalent doses of genomic DNA or saline vehicle alone. Normal guinea pig serum showed similar inhibition of hemolytic complement after in vitro treatment in a PS-oligo dose-dependent manner (Fig. 1).

Human normal donor blood collected into vacutainers previously spiked with PS-oligo for final concentration of 400 μg/ml in whole blood demonstrated no visible blood clotting after up to 18 h incubation at room temperature, as compared to blood collected into vacutainers spiked with the same volume of saline vehicle alone which clotted completely by 30 min. Cell-free supernates of such blood samples demonstrated levels of prothrombin fragments (PF1 + 2) by ELISA (Baxter Dade) that confirmed reduced clotting activity in PS-oligo samples (PF1 + 2 = 12 nM) compared to saline controls (PF1 + 2 > 400 nM). The same supernates exhibited greatly reduced hemolytic complement activities (PS-oligo = 44% of saline control). In parallel, blood samples were collected into 400 μg/ml heparin (porcine, 143 USP K-1 units/mg, Sigma); heparin samples also failed to clot (supernate PF1 + 2 = 14 nM) but hemolytic complement activity was 100% of the saline control. This indicated that the observed complement effect of PS-oligo was not simply due to anti-coagulation.

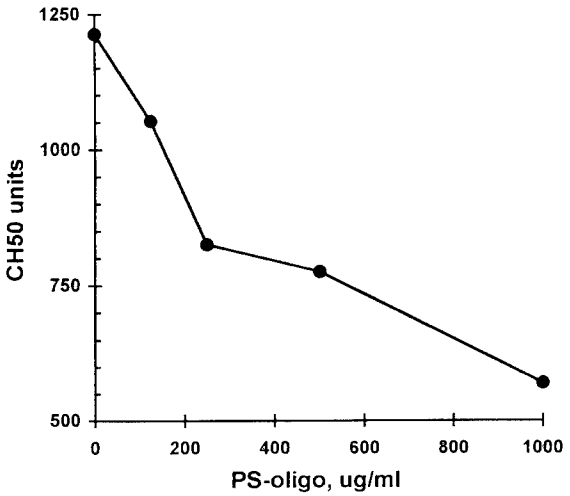


Fig. 1. Hemolytic complement activity (CH50 units) in guinea pig serum following in vitro treatment with varying doses of PS oligo.

The hemolytic complement inhibitory activity of PS-oligo in vitro correlated with serum levels of complement fragment C4d but not with serum fragment Bb (both assayed by Quidel ELISA kits) (Fig. 2). These results suggest that PS-oligo activates the classical (C4d-specific) pathway of complement, thereby consuming the common terminal complement components needed for expression of either classical or alternative hemolytic activity in vitro. Additional experiments are ongoing to confirm this conclusion and to elucidate possible mechanisms.

In vitro studies similar to the above were conducted with modified antisense oligonucleotides with structural differences from the PS-oligo. Serum treated with a PS-oligo of randomized nucleotide sequence demonstrated equivalent anti-complement activity, whereas a phosphodiester oligonucleotide with the same sequence as the PS-oligo produced no measurable anti-complement activity, suggesting that anti-complement activity is independent of nucleotide sequence but dependent upon PS-modification. Serum treated with *Chimeric* oligonucleotide containing the same 25-mer sequence as GEM-91 but four methylphosphonate linkages at both 3′ and 5′ ends, demonstrated much less-potent inhibition of hemolytic complement. Serum treated with *Self-stabilized* oligonucleotide [11,12] containing an extended 3′ self-annealing hairpin-loop sequence, showed anti-complement activity intermediate to that of the linear PS-

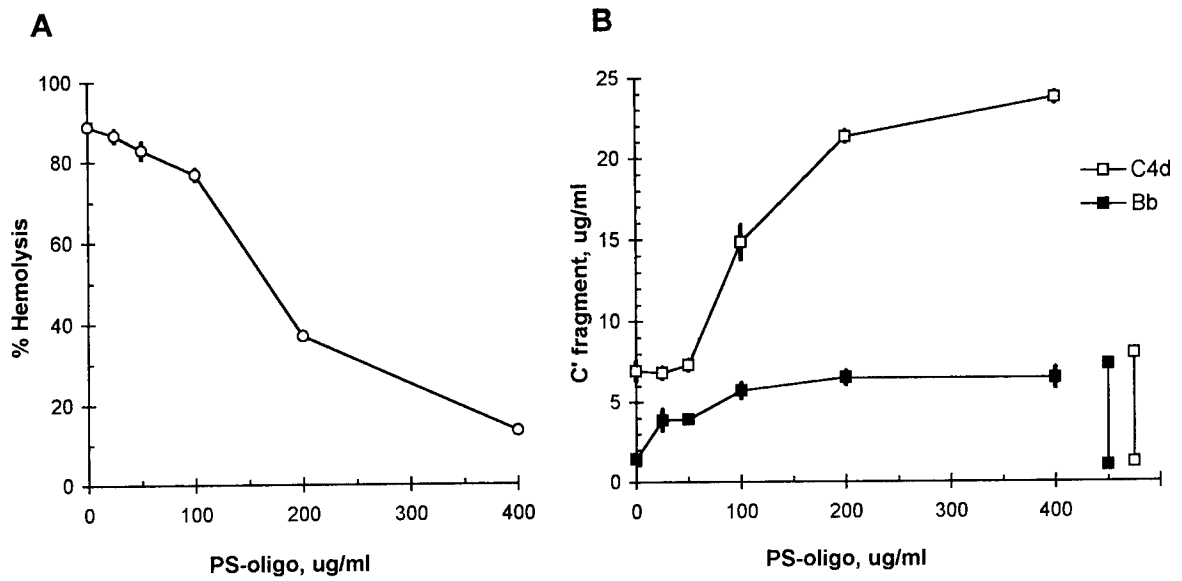


Fig. 2. Correlation of human normal donor serum complement hemolytic activity with generation of complement component fragments following in vitro treatment with varying doses of PS-oligo. (a) Hemolytic activity expressed as % of maximum lysis. (b) ELISA quantitation of fragment C4d (□) and fragment Bb (■); vertical lines at right indicate mean plus 2 S.D. range of fragment concentrations in untreated normal serum. Error bars indicate S.D.

oligo and *Chimeric* oligonucleotide. Serum treated with a *Hybrid* oligonucleotide [13–15] containing four 2′-*O*-methylribonucleoside instead of deoxyribonucleoside residues at both 3′ and 5′ ends, exhibited somewhat lower anti-complement activity as compared to the PS-oligo. Similar results were obtained when treated plasma samples were analyzed for coagulation by standard activated partial thromboplastin clotting time (PTT) assay (Table 1).

In conclusion, PS-oligos have the potential to exhibit dose-dependent acute side-effects related to sequence-independent inhibition of circulating complement and coagulation activities. These effects can be minimized by slow intravenous administration of PS-oligo in monkeys [6]. The therapeutic index of such antisense oligonucleotides may be enhanced by chemical modifications, as supported by the above experimental data. *Chimeric* oligonucleotide [16], containing 17 phosphorothioate and 8 methylphosphonate linkages with the same nucleotide sequence as the 25-mer PS-oligo, exhibited the least effect on either complement or coagulation in vitro. *Self-stabilized* oligonucleotide [11,12], with an 8 phosphorothioate deoxyribonucleotide sequence added to the 3′ end of PS-oligo to form a double-stranded loop, showed reduced complement ef-

Table 1
Serum complement and plasma clotting activities following in vitro treatment with 100 μg/ml of modified oligonucleotides

	Complement CH50	PTT clotting time
	Units % reduction	s % increase
PS-oligo	203 (65)	98 (260)
Self-stabilized oligo	306 (47)	>106 (>270)
Hybrid oligo	357 (38)	76 (180)
Chimeric oligo	457 (21)	49 (81)
Control (no oligo)	580 (0)	27 (0)

fect but similar coagulation effect as PS-oligo. *Hybrid* oligonucleotide [13–15] containing the same number of phosphorothioate linkages had less effect on both complement and coagulation than PS-oligo, suggesting that the nature of nucleotides in addition to phosphorothioate residues may also be important. While these modified oligonucleotides show reduced hemostatic effects in vitro, all retain antisense activity and further exhibit increased in vivo stability [11–16].

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Toxicology Letters

Antisense phosphorothioates as antivirals against human immunodeficiency virus (HIV) and hepatitis B virus (HBV)

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Abstract

For the past 10 years we have focused on the development of antisense treatments against viral infections such as human immunodeficiency virus (HIV) and hepatitis B virus (HBV). We have demonstrated that phosphorothioate oligomers have in vitro anti-HIV activities and in vivo anti-HBV activity. In vitro anti-HIV activities of phosphorothioate oligodeoxynucleotides (*S*-oligo) were classified into sequence non-specific (non-antisense) and sequence specific (antisense). We found that toxicity of *S*-oligo could be also classified into the same 2 categories. Since some of non-specific toxicity is dependent on experimental conditions, in each experiment, we should pay attention to minimize non-specific toxicity of the oligomers.

Keywords: Antisense DNA; Antivirals; HIV; HBV

1. Materials and methods

1.1. Human immunodeficiency virus (HIV)

In vitro assay systems we used were (1) cytopathic effect inhibition assay and (2) viral gene expression inhibition assay. In the first assay we used the HIV-uninfected cell line, ATH8, which is transformed and immortalized with the infection of human T-lymphotropic Virus Type I (HTLV-I). ATH8 cells are very sensitive to the cytopathic effect of HIV and most of cells die within a week after exposure to HIV. In the second assay, we used human T cell line, H9, which is sensitive to the infection of HIV but can survive and become chronically HIV-infected H9 cells.

1.1.1. Cytopathic effect inhibition assay

ATH8 cells were centrifuged, pelleted and exposed to HIV_{IIIb} for 1 h (500 virions per cell). Complete medium, RPMI1640 containing 15% fetal calf serum (FCS) and recombinant interleukin-2 (20 units per ml) were used with various concentration of the oligomers added. The number of viable cells was counted in a hemocytometer using the trypan blue dye-exclusion method on day 7 following exposure to the virus.

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1.1.2. Viral gene expression inhibition assay

To test whether an oligomer has antisense activity on HIV viral expression, we used chronically HIV-1-infected H9 cells ($H9/III_b$). $H9/III_b$ cells were washed extensively to remove the previously produced virions from the medium. After washing, 1250 $H9/III_b$ cells per well in a 96-well culture plate were cultured in the presence or absence of oligomers in 200 μl of the complete medium (RPMI1640 + 15% FCS). Culture supernatants were collected on day 5 and assayed for p24 gag protein by using RIA or ELISA. The cells were pulsed with [^{3}H]thymidine and harvested next day to assess cytotoxicities of oligomers.

1.1.3. Reverse transcriptase (RT) assay

We reported previously the RT assay [1] used and describe it only briefly. Oligo(dT)-poly (rA) was used as primer-template in the presence of [^{3}H]thymidine triphosphate. As a control, oligo(dT)-poly(dA) was used. Phosphorothioate oligodeoxynucleotides (*S*-oligo) or *n*-oligo at various concentrations was added to the assay system.

1.1.4. Oligodeoxynucleotides used

We employed the initiation site of *rev* as the target site of HIV gene with reasons such as conservativeness of the sequence and importance in the viral replication. As controls, *S*-oligo with sense and random sequence and *n*-oligo with antisense sequence were tested.

1.2. Hepatitis B virus (HBV)

HBV has been know to be a very difficult virus for in vitro infection studies. We therefore employed an animal model using HBx transgenic mice. In HBx transgenic mice, histological precancerous changes, including vacuolation and altered foci in the liver, were followed by the development of hepatocellular carcinoma at 1 year of age or older. Because of the insertion of the HBV transactivator gene, HBx, these precancerous changes occurred.

1.2.1. Transgenic mice

Production of HBx gene transgenic mice was reported previously [2]. We used male mice from the H9 strain of HBx transgenic mouse which was homozygous for HBx gene. Mice were maintained and cared for in accordance with the guidelines established by the National Institutes of Health.

1.2.2. Oligodeoxynucleotides used

Two sets of sense and antisense *S*-oligos were designed from the standpoint of base composition (rich in GC) and/or secondary stucture. One set, S-1 (sense) and AS-1 (antisense), was aimed to the GC-rich region of HBx gene and a second set, S-2 and AS-2, was designed to cover the initiation site. Both oligomers were 27-mer and synthesized in the amount of hundreds of milligrams. Sequences of the oligomers will be published elsewhere [3].

1.2.3. RNA extraction, RT-polymerase chain reaction (PCR) and histological studies of tissue

RNA was extracted from liver tissues as described by Chomczynski and Sacchi [4]. RNA was analyzed by RT-PCR using a set of primers [3]. Tissue sections fixed in 10% neutral-buffered formalin were used for hematoxylin and eosin staining.

2. Results

2.1. Anti-HIV activities

We found that *S*-oligo could exhibit 2 different anti-HIV activities. One is sequence-non-specific and another is sequence-specific anti-HIV activity. Sequence-non-specific activity was found in the cytopathic effect inhibition assay where even homo-oligomer *S*-oligo could inhibit de novo infection (no HIV DNA synthesis) possibly with multiple mechanisms including inhibition of HIV RT. On the other hand, the result obtained from the viral gene expression assay was sequence-specific anti-HIV activity, namely antisense activity.

In the enzyme assay for HIV RT, *S*-oligo

showed much more potent inhibition compared to *n*-oligo. Such inhibition, however, could be reversed by addition of bovine serum albumin (BSA) in the assay solution suggesting that the inhibition of RT activity by *S*-oligo is a result of non-specific protein binding. Similarly, increase of percentage of FCS in the complete medium eliminated the cytotoxicity of *S*-oligo whereas a higher percentage than 30% of FCS is somehow toxic and, under such conditions, FCS did not compete with the cytotoxicity of *S*-oligo (data not shown).

In the viral gene expression assay, *n*-oligo showed a profound decrease of [^{3}H]thymidine uptake without decrease of viable cell number. This pseudo-cytotoxicity was derived from competition of non-radioactive thymidine from *n*-oligo degraded by nucleases in the FCS. *S*-oligo is known to be nuclease-resistant, but could be degraded very slowly in culture media and also in vivo. One should take account of not only the oligomers but also the metabolites for toxicity.

2.2. Anti-HBV activity

The data from in vivo experiments using HBx transgenic mice will be published elsewhere. Therefore, we describe the results here briefly without figures and tables.

2.2.1. Down-regulation of HBx gene expression

As an initial trial, we injected 1 of 4 *S*-oligos i.p. into the mice for 7 consecutive days (1 mg/day) at the age of 3 month old. On the last day of injection, mice were sacrificed and gene expression of HBx in liver was examined by means of the RT-PCR. We found a significant decrease of HBx gene expression in AS-2-treated mice whereas other oligomers did not cause a decrease (data not shown).

2.2.2. Long-term treatment with AS-2

We started the treatments before the mice were 3 months old. At 1 week old, even 0.2 mg/day injection 3 times a week was found to be highly toxic to mice, which resulted in death at 4 weeks old. Then we delayed the initiation of the treatment to 2 weeks old and reduced the dosage. With this protocol, the treatment was continued until the mice were 10 weeks old. Significant reduction of the expression and histologically milder changes were found in mice treated with AS-2 but not in mice treated with S-2 or PBS. In a series of treatments, cell infiltration into liver was found in mice treated with AS-1 and S-1 suggesting that toxic reaction could be sequence specific or base composition of oligomers (GC-rich) (data not shown).

3. Discussion

Toxicological consideration of oligomers could not be obtained without knowing more about the characteristics of the experiments. We should compare experiments with each other in terms of their advantages and disadvantages. In vitro assay systems which we used have advantages over in vivo experiments in the following ways: (1) a relatively small amount of oligomer is required; (2) because of relative ease, many samples including several controls can be tested simultaneously; (3) because of relatively defined and short-term assay conditions, experiments can be repeatable in a short time and the data obtained can be reproduced. There are also significant disadvantages as follows: (1) in vitro assay systems have artificial conditions such as use of particular immortalized cell lines, artificially conditioned medium containing FCS, relatively rapid cell growth (possibly different sensitivity to cytotoxicity of compounds) and/or lack of heterogenous cell populations; (2) a larger or smaller amount of viral production in comparison to in vivo situations; (3) lack of pharmacokinetics and tissue distribution. Since in vivo HIV experiments using animal models are neither definitive nor easy, we have been performing in vitro assays for detection of antisense activity. We have found at least several mechanisms of in vitro anti-HIV activities, which were initially confusing and made the antisense strategy perhaps less appealing to some of the researchers. Several mechanisms in anti-HIV activity might be also true in toxicity of oligo-

mers (at least 2: sequence-non-specific and sequence-specific toxicity). In monocytes, *S*-oligo could introduce superoxide (unpublished result), which could be partially reduced by indomethacin. *S*-oligo could also introduce the viral expression of HIV in dormant HIV-infected cells (unpublished data).

Although suitable in vitro assays should be generally employed for antisense studies at the start, in vivo experiments using animal models could sometimes be better in sequence specificity and interpretability of data including toxicity. If enough oligomer is available, one might want to perform in vivo experiments without in vitro assays.

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Molecular aspects of carboxylesterase isoforms in comparison with other esterases

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Abstract

The involvement of carboxylesterase, acetylcholinesterase, butyrylcholinesterase and cholesterol esterase in pharmacology and toxicology are well recognized. However, there are few papers concerning the comparative studies of these serine hydrolases in terms of molecular level. Recently, we have studied various aspects of carboxylesterases using cDNAs of carboxylesterase isozymes purified from 9 animal species and human liver microsomes, and found that there is high homology of the *N*-terminal amino acid sequences of the isozymes tested. On the other hand, we compared the amino acid sequences at the active site of the individual esterases and found that the sequences of all esterases tested are strictly conserved. These results strongly suggest that the esterases involved are classified into the serine hydrolase super family.

Keywords: Carboxylesterase; Cholinesterases; Drug metabolizing enzyme; cDNA cloning

1. Introduction

Carboxylesterases (EC 3.1.1.1), acetylcholinesterase (EC 3.1.1.7), butyrylcholinesterase (EC 3.1.1.8), cholesterol esterase (EC 3.1.1.13) and neuropathy target esterase (formerly neurotoxic esterase, no EC number) are well recognized as playing an important role in toxicology, pharmacology, biochemistry and clinical medicine. Recently, molecular aspects of studies of these esterases such as molecular cloning have been extensively reported, and these results suggest that they have high homology in terms of molecular properties in the light of 3-dimensional structure [1–4]. Despite rapid progress in esterase studies in the past decade, the number of papers concerning the comparison of biochemical and toxicological implications of these esterases is very limited. Thus, substrate specificity of these esterases is mostly overlapping and it is rather difficult to identify which esterase is involved in the toxicity and efficacy of drugs.

Although these esterases hydrolyze vastly different substrates, substantial sequence homology was recognized between well-studied members of the acetylcholinesterase family and the class of other esterases. These proteins contain approximately 550 amino acid residues. A high degree of similarity was detected predominantly in the N-terminal half of the molecule, especially in the region encompassing the active-site serine. Simi-

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larity was less evident in the C-terminal half of the molecule. The most comprehensive studies were carried out by Doctor et al. [3] and Krejci et al. [4] and comprised 16 and 17 different sequences, respectively. These sequences included, in addition to several vertebrate acetylcholinesterases, members of the closely related butyrylcholinesterase family, as well as insect acetylcholinesterases, which display properties intermediate between vertebrate acetylcholinesterase and butyrylcholinesterase [5]. Other sequences represented various other esterases such as cholesterol esterase and carboxylesterases and 3 proteins devoid of known catalytic function.

The mammalian carboxylesterases represent a multigene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing xenobiotics to their respective free acids. As such, they have been implicated in both the detoxication and the metabolic activation of drugs, environmental toxicants and carcinogens [6]. In fact, we showed that changes in liver microsomal carboxylesterase occur during hepatocarcinogenesis [7]. Recently, we reported that rat liver microsomes contain 3 isozymes of carboxylesterase, named RL1, RL2 and RH1. These 3 carboxylesterase isozymes differ considerably from each other in response to hormone treatment, inducibility, substrate specificity, and immunological properties. Carboxylesterase isozymes purified from liver microsomes of several animal species, i.e., mice, hamsters, guinea pigs, rabbits, beagle dogs, pigs, cows, cynomolgus monkeys, rhesus monkeys and humans were compared with 3 isozymes of rat RL1, RL2, and RH1 in terms of substrate specificities, immunological properties, N-terminal amino acid sequences, and cDNA cloning and analysis.

2. Comparative studies on the substrate specificities of liver microsomal isozymes in various mammals and humans

Table 1 shows the comparison of liver microsomal carboxylesterase activities in various mammals and humans. Guinea pig was found to have the highest specific activity of liver microsomes towards *p*-nitrophenylacetate (PNPA) and isocarboxazid among the animal species tested. The highest activity of butanilicaine hydrolase was found in pig liver microsomes.

Rat liver microsomes were found to have the highest specific activity towards malathion. On the other hand, human liver microsomes was

Table 1
Comparison of liver microsomal carboxylesterase activities in various animal species and humans

Species	Specific activity			
	PNPA	MALA	BUTA	ISOC
Rat (5)	1.93 ± 0.1	74.6 ± 9.2	0.12 ± 0.03	2.00 ± 0.4
Mouse (5)	8.52 ± 0.5	14.0 ± 2.4	0.01 ± 0.01	2.63 ± 0.2
Hamster (5)	14.9 ± 1.1	26.4 ± 3.4	0.58 ± 0.07	8.30 ± 1.0
Guinea pig (5)	19.0 ± 4.6	41.4 ± 3.5	0.04 ± 0.01	29.5 ± 2.6
Rabbit (2)	10.3	59.9	1.08	6.57
Pig (1)	10.9	28.8	1.62	4.17
Cow (1)	8.77	<0.002	1.11	1.61
Dog (1)	2.42	26.7	1.06	2.29
Monkey (3)	4.37 ± 0.32	42.5 ± 2.5	<0.005	5.63 ± 0.8
Human (13)	1.95 ± 0.39	116 ± 15.8	0.13 ± 0.03	1.05 ± 0.3

[a] PNPA, *p*-nitrophenylacetate hydrolase (μmol/mg/min); MALA, malathion hydrolase (nmol/mg/min); BUTA, butanilicaine hydrolase (μmol/mg/min); ISOC, isocarboxazid hydrolase (nmol/mg/min).

found to have the lowest activity towards all substrates used. These marked interspecies variations of carboxylesterase activities may be explained, at least in part, by the differences in the properties of the various carboxylesterase isozymes.

We already reported [8] that the 3 rat liver carboxylesterase isozymes (RL1, RL2, RH1) were immunologically different from each other as determined by immunoblotting analysis. Rat, mouse, hamster and guinea pig liver microsomes contained a single polypeptide (60 KDa) staining with anti-RL1 antibody, and the position of migration was the same in every case. Anti-RL2 antibody showed immunocross-reactivity only with rat liver microsomes. Microsomes from rat, mouse, hamster, rabbit, beagle dog, pig, cow, cynomolgus monkey, and human livers gave a single band (60 KDa) with anti-RH1 antibody, though guinea pig liver microsomes gave 2 bands. These results suggest that 9 mammals and humans have similar carboxylesterase isozymes to rat RH1.

3. Purification of carboxylesterase isozymes from liver microsomes of various mammals

Table 2 shows that the purified preparations have similar subunit molecular weights (57–64 KDa), but their isoelectric points ranged widely from 4.7 to 6.5. Since most of the isozymes studied were bound to Con-A affinity column, and eluted from the column with an α-methylmannoside, these carboxylesterases are glycoproteins bearing a sugar moiety which contains high mannose type. Table 1 also shows the catalytic activities of the highly purified carbox-

Table 2
Comparison of physicochemical properties of carboxylesterase isozymes

Species	Isozymes	Subunit weight	pI	Association form	ConA affinity
Rat	RH1	58 KDa	6.0	Trimer	+
	RL1	61 KDa	6.5	Monomer	+
	RL2	61 KDa	5.5	Monomer	+
	RL3	61 KDa	5.0	Monomer	+
	RL4	61 KDa	4.6	Monomer	−
	RLlec	60 KDa	4.7	Monomer	−
Mouse	MH1	60 KDa	5.8	Trimer	+
	ML1	59 KDa	5.9	Monomer	+
	ML2	60 KDa	5.1	Monomer	+
	ML3	60 KDa	4.7	Monomer	−
Hamster	H1	58 KDa	5.7	Trimer	+
	HL1	58 KDa	6.0	Monomer	+
Guinea pig	GPH1	57 KDa	5.3	Trimer	+
	GPL1	64 KDa	5.9	Monomer	+
	GPL2	61 KDa	5.6	Monomer	+
Rabbit	RB1	62 KDa	5.5	Trimer	+
Dog	D1	60 KDa	5.0	Trimer	+
Pig	P1	58 K–62 KDa	5.2–5.4	Hetero trimer	+
Cow	B1	59 KDa	6.0	Trimer	+
Monkey	MK1	60 KDa	5.5	Trimer (?)	+
(Cynomolgus)	MK2	63 KDa	4.7	Monomer	+
Monkey	RMK1	60 KDa	5.6	Trimer (?)	+
(Rhesus)	RMK2	63 KDa	4.7	Monomer	
Human	HU1	61 KDa	5.6	Trimer	+
	HU2	61 KDa	5.4	Trimer	+
	HU3	61 KDa	4.3	Monomer	+

```
RH1          Y-P-S-S-P-P-V-V-N-T-V-K-G-K-V-L-G-K-Y-V-
RL1          D-P-S-S-P-P-V-V-D-T-V-K-G-K-V-L-G-K-Y-V-S-L-
RL2          X-P-S-X-P-P-V-V-N-X-V-K-G-K-V-L-G-K-Y-V-
HL1          A-P-S-S-P-P-V-V-N-X-V-K-G-K-V-L-G-
H1           S-P-S-X-P-X-V-V-D-X-V-
GPL1       X-X-P-S-X-P-X-V-V-D-X-K-Y-G-K-V-L-G-K-Y-
RB1          H-P-S-X-P-P-V-V-N-X-V-K-G-K-V-L-G-K-Y-V-
D1           Y-P-S-X-P-P-V-V-N-X-V-K-G-K-V-L-G-K-Y-V-
P1         G-E-P-A-V-P-P-V-V-D-T-A-Q-G-X-X-L-G-K-Y-
B1           L-A-V-S-P-P-I-V-D-X-A-Q-G-X-V-L-G-K-L-V-
MK1          G-P-S-S-P-P-V-V-D-D-V-K-G-K-V-L-G-K-
MK2          K-S-A-S-P-X-V-
HU1          G-P-P-S-P-P-V-V-D-D-T-X-G-K-X-L-
```

Fig. 1. Amino-terminal sequences of carboxylesterase isozymes from rat (RL1, RL2, RH1), hamster (H1, HL1), guinea pig (GLP1, GLP2), rabbit (RB1), beagle dog (D1), pig (P1), cow (B1), cynomolgus monkey (MK1, MK2), human (HU1).

ylesterase isozymes from liver microsomes of various animal species, towards PNPA, malathion, butanilicaine, isocarboxazid, and palmitoyl-CoA. The isozymes of hamster (H1), guinea pig (GPL1 and GPH1) and rabbit (RB1) possess higher hydrolytic activities towards PNPA; those of mouse (MH1), hamster (H1) and rabbit (RB1) have the highest activities toward butanilicaine. Hydrolysis of isocarboxazid was catalyzed most effectively by guinea pig GPL1, and rat RL1 showed the highest catalytic activity toward palmitoyl-CoA.

Fig. 1 shows the amino-terminal amino acid sequences of carboxylesterase isozymes of various mammalian species. It is of interest to note that the isozymes tested show high homology in this region, except for monkey MK2, but the first amino acid in the sequence is different in all isozymes tested.

4. Species differences in the induction of liver microsomal carboxylesterases by peroxisome proliferators

Liver microsomal carboxylesterases are induced by exogenous compounds, i.e., phenobarbital [9], Aroclor 1254 [9], polycyclic aromatic hydrocarbons [9], aminopyrine [9], synthetic glucocorticoid [10], pregnenolone 16 α-carbonitrile [10], clofibrate [9,11], perfluorinated fatty acids [12], and di(2-ethylhexyl)phthalate (DEHP) [13]. Table 3 shows the differences in the capability of perfluorinated fatty acid and DEHP, which are used in industry, to induce liver microsomal carboxylesterase isozymes in rats, mice and hamsters, studied by measuring changes in hydrolytic activities and by evaluating changes in the contents of the carboxylesterase isozymes by immunological assay with specific antibodies. Perfluorooctanoic acid (PFOA), perfluorodecanoic acid (PFDA), perfluorooctane sulfonic acid (PFOS) and 1-H,1-H-pentadecafluorooctanol (PFOL) were used as perfluorinated fatty acid. The administration of DEHP, PFOA, PFOS and PFOL markedly increased the hydrolytic activity towards PNPA, isocarboxazid, butanilicaine in rats and mice and the contents of carboxylesterase isozymes, but not in hamsters. And, the administration of DEHP also markedly increased the hydrolytic activity toward palmitoyl-L-carnitine (6.5-fold) in C57/BL mouse liver microsomes, which was strongly inhibited by mouse ML3-IgG; whereas, the increased butanilicaine hydrolase activity was not inhibited

Table 3
Effects of peroxisome proliferators on liver microsomal carboxylesterases in various mammalian species

Species	Strain	CPIB	DEHP	PFDA	PFOA	PFOL	PFOS
Rat	SD	↑	↑↑	↓	↑↑	↑	↑
	F344	↑			↑		
	LEW	=			=		
Mouse	C57BL	↑↑	↑↑↑↑	↑↑	↑↑↑	↑↑	↑
	DBA	↑↑	↑↑↑	↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑
Hamster	Syrian	↑	↑	↓	↑	↑	↑
Guinea pig	Hartley	=		↓	=	=	=

=, no change; ↑, 1.2–2.5X; ↑↑, 2.5–5.0X; ↑↑↑, 5.0–10.0X; ↑↑↑↑, 10.0X; CPIB, clofibrate.

Table 4
Comparison of conserved motifs in carboxylesterase, cholinesterase and cholesterol esterase

	Cys(98)	Gly-Gly(124-125)	Scr(203)	Glu(336)	His(450)
HU1	SEDCLYLNI	WIHGGGLMVGAA	TIFGESAGG	NKQEFGW	G-DHGDE
RH1	SEDCLYLNV	WIHGGGLVVGGA	TIFGESAGG	NKQEFGW	G-DHGDE
RL1	SEDCLYLNI	WIHGGGLTQGGA	TIFGESAGG	NKQECGW	G-DHADD
RS1	SEDCLYLNI	WIHGGGLIIGGA	TIFGESAGG	NKQEFGW	G-DHGDE
MH1	SEDCLYLNI	WIHGGGLVVGGA	TIFGESAGG	NKQEFGW	G-DHGDE
MS1	SEDCLYLNI	WIHGGGLVIGGR	TIFGESSGG	NKQEFGW	G-DHGDE
Rabbit1	SEDCLYLNI	WIHGGGLMVGGA	TIFGESAGG	NQQEFGW	G-DHGDE
Rabbit2	SEDCLYLNI	WIHGGGLTMGMA	TIFGESAGG	NNDEYGW	A-DHGDE
AT51	SEDCLYLNI	WIHGGALVMGMA	TIFGVSAGG	DSDECGW	A-DHGDH
HUM-ACHE	SEDCLYLNV	WIYGGGFYSGAS	TLFGESAGA	VKDEGSY	GVPHGYE
HUM-BCHE	SEDCLYLNV	WIYGGGFQTGTS	TLFGESAGA	NKDEGTA	GVMHGYE
TC-ACHE	SEDCLYLNI	WIYGGGFYSGSS	TIFGESAGG	NKDEGSF	GVIHGYE
HUM-CHOL	DEDCLYLNI	WIYGGAFLMGSG	TLFGESAGG	NNMDGHI	GADHADD
RAT-CHOL	QEDCLYLNI	WIYGGAFLMGSG	TIFGESAGA	NDMDGHL	GADHADD

by anti-ML3 IgG, and the activity was strongly inhibited by anti-rat RL1 IgG. It is of interest to note that at least 2 carboxylesterase isozymes, with different functions, were induced by peroxisome proliferators.

5. Comparison of conserved motifs in carboxylesterase, cholinesterase and cholesterol esterase

It is well recognized that amino acid composition at the active site of esterases are Ser, Glu, His. The conserved motifs of carboxylesterases were compared with those of acetylcholinesterase and cholesterol esterases. As shown in Table 4, Cys(98), Gly-Gly(124-127), Ser(203), Glu(336) and His(450) are highly conserved in most of the isozymes, except E was replaced by D(136) of HUM-Chol and Rat-Chol. The similarities of the conserved motifs of these esterases seem to be closely associated with the overlapping of their substrate specificities.

6. Comparison between nucleic acid sequence and the deduced amino acid sequences of rat and human carboxylesterases

In the present study, a λ gt11 library from peroxisome proliferator-treated rat liver was screened with antibody raised to a purified rat liver carboxylesterase RL1, which is highly specific for long chain acyl-CoA hydrolase, and also screened with antibiody raised to RH1. The nucleotide sequence of 3′-end of the clone included an open reading flame terminating with stop codon (TAG), followed by an untranslated region including a polyadenylation signal (AATAAA), and a 17-bp poly (A) tail. The

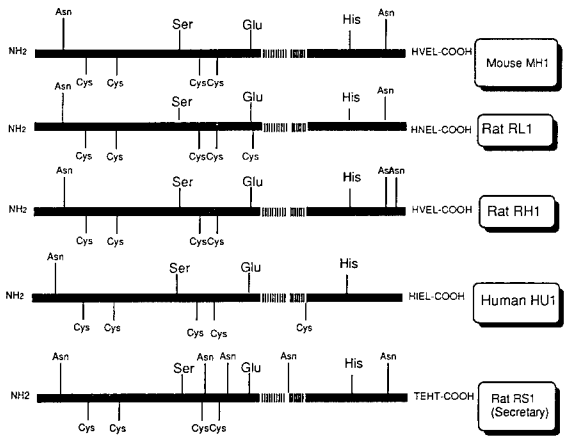


Fig. 2. Diagrammatic comparison of deduced amino acid sequences of rat, mouse and human carboxylesterase isozymes.

deduced amino acid sequence of the clone possesses many structural characteristics that are highly conserved among rat pI 6.1 [11], E1 (secretary type) [14], and human hCE [15], including the active site sequence of the serine residue (GESAGG), and 4 cysteines which may be involved in the specific disulfide bond. Fig. 2 shows the diagrammatic comparison of the deduced amino acid sequence of rat (RL1, RH1 and RS1, secretary type) and human HU1. It is well known that proteins which are retained in the ER lumen contain the retention signal at their -COOH terminus of the tetrapeptide (KDEL-COOH) [16]. Carboxylesterase contains HXEL-COOH as the terminal ER-retention signal.

7. Sequence identities of the 14 esterases/lipases

Table 5 summarizes the sequence identities of carboxylesterase, acetylcholinesterase, butyrylcholinesterase and cholesterol esterase. These 4 esterases are markedly similar in terms of the characteristics such as substrate specificity. The nucleotide sequences of the isozymes of these esterases are highly homologous. Based on the high homology and similarity of the characteristics, we tried to classify carboxylesterase isozymes into 2 groups, named CES 1 and CES 2. CES 1 contains HU1 and other 6 isozymes, and CES 2 consists of Rabbit 2 and AT 51. Acetylcholinesterase, butyrylcholinesterase and cholesterol esterase have only 30% homology with HU1.

8. Conclusion

Liver microsomal carboxylesterases in mammals and humans play an important role in drug and lipid metabolism in the ER, and it is noteworthy that the isozymes from various animal species and humans examined here showed considerable similarities in physical and immunological properties. The amino acid sequences at the N-terminals and the active site of the carboxylesterase isozymes are highly homologous, but not similar in substrate specificities. The reason for the interspecies variation may be, at least in part, due to the difference in the amino acid sequences of substrate binding site.

Based on the experimental data using cDNA clones of various carboxylesterases in our laboratory, deduced amino acid sequences of carboxylesterase isozymes were compared with those of acetylcholinesterase, butyrylcholinesterase and cholesterol esterase which were reported in the literature. It is of interest that the sequences required for the hydrolytic capability at the active site of all of these esterases, including carboxylesterase, are highly conserved. This is a common structure of serine enzyme families

Table 5
Sequence identities of the 14 esterase/lipase

Enzyme name	Gene symbol	Trivial name	Species	Homology (%)
Carboxylesterase	CES1	HU1	Human	100.0
		MH1	Mouse	78.5
		RH1	Rat	78.4
		Rabbit 1	Rabbit	77.3
		RL1	Rat	68.2
		RS1	Rat	66.9
		MS1	Mouse	64.0
	CES2	Rabbit 2	Rabbit	45.8
		AT51	Hamster	44.0
Acetylcholinesterase			Human	29.3
T. californica			29.1	
Butyrylcholinesterase			Human	30.2
Cholesterol esterase			Human	29.5
			Rat	28.0

which are responsible for the hydrolysis of endogenous and exogenous compounds. Finally, the studies on the physiological role of the carboxylesterases at the molecular level can begin to be studied. Thus, this report is an initial attempt to compare the characteristics of individual esterases in terms of the respective cDNAs.

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Toxicology Letters

Catalytic properties and distribution profiles of paraoxonase and cholinesterase phenotypes in human sera

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Abstract

Paraoxonase activities (322 healthy subjects) measured in the absence of ethylenediaminetetraacetic acid (EDTA) had a polymodal distribution profile with 60% of the subjects in the low activity mode; the activity measured in the presence of EDTA had a unimodal skewed distribution. Cholinesterase (ChE) activities (365 healthy subjects) had a unimodal, slightly skewed distribution. Patients with dementia (74) and patients with hyperlipidaemia (159) had different median paraoxonase and ChE activities than healthy subjects and all activity profiles had a higher skewness. The ChE variants usual (UU), fluoride resistant (FS) and atypical (AA) had the same affinity for the studied charged and uncharged ligands. The variants differed in rates of inhibition by the charged organophosphates and carbamates.

Keywords: Human serum paraoxonases and cholinesterases; Phenotypes; Activity distribution profiles; Relation to diseases; Interaction with ligands

1. Introduction

Two groups of esterases react with organophosphorus compounds: phosphoric triester hydrolases and serine esterases. Both groups act on the same ester bond and both react only with organophosphorus compounds which have no free -OH group on the phosphorus. Phosphoric triester hydrolases have only recently been classified and they have a common classification number (EC 3.1.8) [1]. Serine esterases have no common classification number and the group includes carboxylic ester hydrolases (such as acetylcholinesterase EC 3.1.1.7, butyrylcholinesterase EC 3.1.1.8 and carboxylesterase EC 3.1.1.1) and serine endopeptidases (such as trypsin EC 3.4.21.4 and chymotrypsin EC 3.4.21.1) [1].

Organophosphorus compounds are substrates of phosphoric triester hydrolases and inhibitors of serine esterases (cf. [2–4]). Phosphorylation of acetylcholinesterase is the main cause for their toxicity. Butyrylcholinesterase and carboxylesterase are also phosphorylated by the organophosphates acting thereby as 'scavangers' for these compounds. Phosphoric triester hydrolases are also detoxifying enzymes.

The phosphoric triester hydrolases (EC 3.1.8.1 and EC 3.1.8.2) are defined as enzymes which act on organophosphorus compounds (including es-

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ters of phosphoric and phosphinic acids) and on phosphorus anhydride bonds; they require divalent cations for activity and are inhibited by chelating agents [1]. The 2 phosphoric triester hydrolases are often named paraoxonase and DFPase after their respective substrates, paraoxon and diisopropyl fluorophospate (DFP). Some enzyme sources contain 2 phosphoric triester hydrolases: one inhibited by chelating agents and the other insensitive to chelating agents. This is also the case with the paraoxonases in human sera: one is inhibited by ethylenediaminetetraacetic acid (EDTA) and the other is insensitive to EDTA. The existence of phosphoric triester hydrolases insensitive to chelating agents might require a broader description of their catalytic properties in the Enzyme Nomenclature.

This paper deals with the distribution of paraoxonase (EC 3.1.8.1) and cholinesterase (ChE; EC 3.1.1.8) activities, and their phenotypes, in a population group from Zagreb, Croatia, and with some catalytic properties of these 2 esterases. Activities and distribution profiles are also discussed in relation to certain diseases.

2. Paraoxonases

Paraoxonase activities were measured in a group of healthy individuals in the presence and in the absence of EDTA, and the results are given in Table 1. Under the applied experimental conditions at pH 7.4 [5,6], the contribution of the EDTA-insensitive activity (v-ins) amounted on average to 6.2% of the total activity (v-tot). Because the contribution was small, the v-tot represented well the EDTA-sensitive enzyme (v-sen). When paraoxonase activities are measured at higher pH (10–11) the contribution of v-ins to the total activity is higher [7].

The distribution of v-tot activities was polymodal; only the low activity mode was well separated and it comprised 60% individuals (Table 1). Eckerson et al. [8] found a better separation of modes when v-tot activities were measured in the presence of 1.0 mM NaCl (at pH 10.5) than in the absence of NaCl; under these conditions 3 activity modes were separated, with 46% individuals in the low mode. The percentage of individuals in the low activity mode (obtained from measurements under different experimental conditions) ranged from 45 to 61% as reported from 13 European countries, the USA and Canada [7].

The distribution profile of v-ins activities was unimodal but skewed (Table 1). The contribution of v-ins to the v-tot activity was greater in the low activity mode (average 13%) than in the high activity mode (average 1–2%). No correlation was found between the v-ins and v-sen hydrolysis of paraoxon ($r = 0.1$–0.4; [5,6]) confirming that paraoxon was hydrolysed by 2 different enzymes in human sera [7].

Studies on the substrate specificity of paraoxonases, concerning their interaction with carboxylic acid esters, have so far failed to result in any firm conclusion. Eckerson et al. [8] obtained a high correlation coefficient between the rates of hydrolysis (v-tot) of phenylacetate and para-

Table 1
Paraoxonase activities in serum samples from 322 individuals from Zagreb, Croatia

	v-tot (mmol min^{-1} l^{-1})	v-ins
Median	0.120	0.00741
Range	0.028–0.767	0.00077–0.0322
Distribution profile	Polymodal	Unimodal
Skewness coefficient	1.2	0.76
Nadir	0.180	–
Individuals in low act. mode	60%	–

Females = 151; males = 171; age = 16–82 years; median age = 34 years. v-tot and v-ins denote activities measured in the absence and presence of EDTA (1.0 mM), in Tris–HCl buffer, pH 7.4, at 37 °C, with paraoxon (5.0 mM) as substrate.

oxon ($r = 0.8–0.9$), indicating that the 2 substrates were hydrolysed by the same enzyme. They attributed it to arylesterase (EC 3.1.1.2), whose characteristic substrate is phenylacetate [1]. Contrary to that, Geldmacher and Diepgen [7] and Reiner et al. [5,6] obtained a low correlation between paraoxon and phenylacetate rates of hydrolysis ($r = 0.1–0.5$) indicating that different enzymes hydrolysed the 2 substrates. However, the EDTA-sensitive rates of hydrolysis of β-naphthylacetate and paraoxon correlated well ($r = 0.85$) [6]. It seems that the substrate specificities of paraoxonases and arylesterases partially overlap.

Phenylacetate and β-naphthylacetate are well hydrolysed by human sera and the bulk of activities can be inhibited by EDTA (average inhibition 99 and 86% respectively, at pH 7.4) [6]. This indicated that each substrate was hydrolysed by 2 enzymes. The v-ins activities were shown to have catalytic properties of a serine esterase, i.e. the activities were inhibited by organophosphorous compound and carbamates (Tabun, iso-OMPA, eserine) and inhibition followed the kinetics of a time-dependent reaction [6].

3. Cholinesterases

ChE activities were measured in a group of healthy individuals using acetylthiocholine (ATCh) and benzoylcholine (BzCh) as substrates (Table 2). The distribution profile of both activities was unimodal, but slightly skewed. Both skewness coefficients were positive numbers which means that the curves were skewed towards higher activities.

The ChE was phenotyped by measuring the inhibition of BzCh hydrolysis with dibucaine, fluoride and (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Ro-02-0683) [9,10]. A group of 441 individuals from Zagreb (which include all subjects from Table 2), had 89% of the usual (UU) phenotype while the other 11% were UA, UF, AF, AK and FF phenotypes (Table 2 and [11,12]). The phenotypes other than UU have lower activities than the UU. The skewness of the ChE profiles towards higher activities therefore can not be attributed to the presence of different phenotypes.

No atypical or silent phenotypes were observed in the above group. However, 4 hospitalized patients, who had been given succinylcholine as a muscle relaxant and developed prolonged apnea, were also phenotyped. In these patients and their family members (total of 24 individuals), 6 were of the atypical (AA) phenotype, 7 were UA and 11 were UU [12].

The interaction of ChE variants with substrates and inhibitors was studied on 3 serum samples belonging to the UU, fluoride resistant (FS) and AA ChE phenotypes (Table 3 and [13,14]). The enzyme/substrate reaction deviated from the Michaelis kinetics. The Hill coefficients ranged from 0.45 to 0.72 with no obvious difference either between the phenotypes or between the 2 substrates, ATCh and PTCh. The same holds for the Michaelis constants which ranged

Table 2
Cholinesterase activities (v) in serum samples from 365 individuals from Zagreb, Croatia

	ATCh (v/mmol min^{-1} l^{-1})	BzCh (v/mmol min^{-1} l^{-1})
Median	2.56	0.80
Range	1.07–4.69	0.33–1.54
Distribution profile	Unimodal	Unimodal
Skewness coefficient	0.38	0.31
Phenotypes	UU UA UF AF AK FF	
Frequency (%)[a]	89 5.9 3.6 0.46 0.46 0.23	

Females = 170; males = 195; age = 16–89 years; median age = 40 years. Activities were measured in phosphate buffer, pH 7.4, at 25°C with ATCh (5.0 mM) and BzCh (0.05 mM) as substrates.
[a] Ref. [13].

Table 3
Kinetic constants for the interaction of UU, FS and AA serum ChE variants with substrates and inhibitors measured in phosphate buffer, pH 7.4, at 25°C

	UU	FS	AA
Substrates			
ATCh K_m	0.56	0.71	0.96
V_{max}	2.9	1.4	1.1
Hill coeff.	0.45	0.57	0.71
PTCh K_m	0.76	0.70	0.57
V_{max}	5.3	2.5	1.5
Hill coeff.	0.64	0.63	0.72
Reversible inhibitors			
Charged: K_i			
HI-6 K_i	0.23[a]	0.56	0.47[a]
PAM-2 K_i	0.88[a]	1.5	1.1[a]
Uncharged: K_i			
4,4′-BP K_i	1.6[a]	1.3	1.8[a]
K_i'	5.7	4.8	3.2
Progressive inhibitors			
Charged:			
Phosphostigmine	38.0[a]	9.7	0.81[a]
Ro 02–0683	8.3[b]	4.7	0.05[b]
Uncharged:			
Paraoxon	3.0[a]	2.4	3.1
VX	2.1[a]	0.67	0.10[a]

Substrates: acetylthiocholine, ATCh; propionylthiocholine, PTCh. Inhibitors: oximes: HI-6 and PAM-2; 4,4′-bipyridine; organophosphates: phosphostigmine, VX and paraoxon; carbamate, Ro 02–0683.
Constants (units): K_m (mM); V_{max} (mmol min^{-1} l^{-1}); enzyme/inhibitor dissociation constants K_i and K_i' (mM); rate constant of inhibition k_i (l μmol^{-1} min^{-1}).
[a] Ref. [14].
[b] Ref. [18].

from 0.56 to 0.96 mM. The V_{max} values for single serum samples of different phenotypes cannot be compared, because activity ranges of phenotypes overlap [9,10].

Dissociation constants for reversible inhibition were calculated from the effect of substrate concentration upon the degree of inhibition (cf. [4]). All 3 inhibitors were competitive reversible inhibitors. HI-6 was a better inhibitor for all 3 phenotypes than PAM-2 or 4,4′-bipyridine (4,4′-BP (Table 3). The inhibition of 4,4′-BP revealed 2 dissociation constants obtained from inhibitor/substrate competition at low (0.02–0.25 mM) and high (1–10 mM) substrate concentrations, indicating 2 binding sites for 4,4′-BP on all 3 ChE variants. The same was shown for binding of 4,4′-BP and the oximes to acetylcholinesterase [15,16]. Inhibition by HI-6 and PAM-2 could be measured only at low substrate concentrations due to the interference of the non-enzymic oxime/ATCh reaction [17]. A given reversible inhibitor had about the same affinity for all 3 ChE variants irrespective of whether the compound was charged or uncharged (Table 3).

Phosphostigmine and Ro 02-0683 are progressive ChE inhibitors. Both compounds have a quaternary nitrogen in the leaving group and both were 1–2 orders of magnitude better inhibitors of the UU than FS or AA phenotypes [13,14,18]. VX, with a tertiary nitrogen in the leaving group (which might be partially protonated at pH 7.4), exhibited the same inhibition pattern. The uncharged paraoxon reacted equally fast with all 3 variants [14].

Positively charged compounds are considered to be better reactants for the UU than the AA ChE phenotype [19]. Our results indicated that a positive charge did not have the same effect on all catalytic steps in the enzyme/ligand reaction (Table 3). Expressed in terms of K_m or K_i, the 2 charged substrates and 2 charged inhibitors had about the same affinity for the 3 ChE variants. However, the difference between variants became very obvious when the reaction was defined in terms of the rate constants of phosphorylation or carbamylation of the enzyme.

4. Paraoxonase and ChE activities related to disease

Many studies have been conducted on serum paraoxonase and ChE activities as possible markers of certain diseases [9,10,20–22]. Studies on serum paraoxonase referred so far only to v-tot activities. We have studied v-tot and v-ins paraoxonase activities, and ChE activities, in patients with dementia and patients with hyperlipidaemia [23,24].

In a group of 74 demented elderly patients (Alzheimer type dementia, multi-infarct dementia, mixed type dementia; above 60 years) the median v-ins and v-tot paraoxonase activities were lower than in a group of non-diseased

individuals of the same age [24]. Cholinesterase activities measured with BzCh as substrate were lower in the demented than in the non-demented group (72 subjects; above 60 years) (activities: 0.61 vs. 0.80) and the activity distribution profiles had a higher skewness coefficient in the demented than in the non-demented elderly (skewness coefficient: 0.91 vs. 0.24). All demented subjects were of the UU ChE phenotype.

In a group of 159 patients with hyperlipidaemia (17–81 years) the median v-ins and v-tot paraoxonase activities were 0.065 and 0.00433 [23] which is lower than in a non-diseased population (Table 1). The distribution profile of v-tot was polymodal with 72% patients in the low activity mode, which is more than in a non-diseased population (Table 1). Cholinesterase activities were measured with propionylthiocholine as substrate (1.0 mM, phosphate buffer pH 7.4, 25°C) in 108 patients and compared to activities obtained in 88 healthy subjects. In patients with hyperlipidaemia both the median serum ChE activity and the skewness of the distribution profile were higher than in the non-diseased group (activities: 5.58 vs. 3.40; skewness coefficient: 0.87 vs. 0.52).

When comparing paraoxonase or ChE activities, one also has to take into account the age and sex of the studied group. Cholinesterase activities are known to vary according to sex and age (cf. [9,10]). Data for paraoxonase activities, concerning sex and age, are ambiguous (cf. [7]). We found no difference concerning sex for the 2 paraoxonases. However, when the group shown in Table 1 was subdivided according to age, the 60–82 years subjects ($N = 46$) had lower v-tot and v-ins activities than the 16–29 years ($N = 109$) or 30–59 years ($N = 167$). A more detailed analysis concerning age would require groups which are equal in size and greater in number.

It follows from these results that dementia and hyperlipidaemia are reflected in paraoxonase and ChE activity distribution profiles and in their median activities. However, the activity ranges between the diseased and non-diseased populations grossly overlapped, and so far it does not seem that a given enzyme activity can be taken as an indicator of either of these diseases.

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Structural bases for the specificity of cholinesterase catalysis and inhibition

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Abstract

The availability of a crystal structure and comparative sequences of the cholinesterases has provided templates suitable for analyzing the molecular bases of specificity of reversible inhibitors, carbamoylating agents and organophosphates. Site-specific mutagenesis enables one to modify the structures of both the binding site and peptide ligand as well as create chimeras reflecting one type of esterase substituted in the template of another. Herein we define the bases for substrate specificity of carboxylesters, the stereospecificity of enantiomeric alkylphosphonates and the selectivity of tricyclic aromatic compounds in the active center of cholinesterase. We also describe the binding loci of the peripheral site and changes in catalytic parameters induced by peripheral site ligands, using the peptide fasciculin.

Keywords: Acetylcholinesterase; Cholinesterase; Serine hydrolase; Organophosphates; Fasciculin; Enantiomeric inhibitors

1. Introduction

Sequencing of the cholinesterases and the cloning of their genes a decade ago revealed that these enzymes defined a family of serine hydrolases distinct from the well characterized pancreatic protease and subtilisin families of enzymes [1]. The diversity in this hydrolase family is not only reflected in enzymes which hydrolyze complex ester structures such as juvenile hormone and lysophospholipids, but the family also includes enzymes with distinct differences in mechanism, the epoxide hydrolases and dehydrohalogenases [2,3]. Even more remarkable is the observation that several proteins without hydrolase function such as thyroglobulin, the tactins and the neuroligins show sequence identity with the hydrolases in this family [3,4]. Solutions of the crystal structures of acetylcholinesterase (AChE) and the fungal lipases revealed a structural motif, termed the α/β hydrolase fold [2,5]. Surprisingly, this motif is also shared by several enzymes that possess no discernible sequence

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identity with the cholinesterases [2,3]. Hence, this family continues to enlarge, and the α/β hydrolase fold emerges as a common structural motif associated with a wide variety of catalytic and surface contact functions.

The catalytic steps in ester hydrolysis involve an initial transacylation step in which the acyl group is transferred from substrate to the serine on the enzyme with the departure of the alcohol moiety of the substrate [3,6,7]. This is followed by water addition with the concomitant deacylation of the enzyme. Of importance is that both acylation and deacylation proceed through tetrahedral transition states which are facilitated by hydrogen bonding from amide backbone hydrogens to the carbonyl oxygen in the oxyanion hole.

The solution of the crystal structure of the enzyme [5] has revealed that the catalytic serine and the accompanying histidine and glutamate in the catalytic triad lie centrosymmetric to each subunit in the molecule at the base of a deep gorge. The crystal structure, sequence comparisons and site-specific mutagenesis now enable one to define distinct residues and discrete domains which contribute to overall specificity for substrates and inhibitors. Since AChE and butyrylcholinesterase (BuChE) show well defined differences in specificity, substitutions can be developed from either template. Table 1 outlines a series of selective and non-selective inhibitors of the cholinesterases and the residues in the enzyme which dictate specificity.

2. Specificity governed by acyl pocket dimensions

The acyl pocket in AChE is outlined by the side chains of two phenylalanines, F_{295} and F_{297}, in the mammalian enzyme; in BuChE the residues at corresponding positions are leucine (L) and isoleucine (I), therein occupying much smaller volumes. Substitution of L for F at the 295 position provides a sufficient reduction in side chain volume to allow butyrylcholine to become an efficient substrate, while substitution of I or V for F at the 297 position changes the kinetic profile of the enzyme from one that shows substrate inhibition to one of substrate activation (Fig. 1, Table 1). Substrate inhibition is characteristic of AChE, whereas substrate activation is characteristic of BuChE [8–10]. Hence, the two phenylalanines outlining the acyl pocket play distinct roles in governing the catalytic profile of the cholinesterases. The carboxylesters possess trigonal, planar acyl groups, and by extending these observations to tetrahedral substrates such

Table 1
Cholinesterase domains affecting ligand specificity

	Substrate	Selective inhibitors	AChE/BuChE preference	AChE residues[a]
1. Acyl pocket (active center)	Critical to acyl group dimensions; influences substrate inhibition	IsoOMPA	BuChE	Phenylalanine-$F_{295(288)}$ Phenylalanine-$F_{297(290)}$
2. Choline subsite (active center)	Specificity neutral versus cationic esters	Ethopropazine Huperzine	BuChE AChE	Tyrosine-$Y_{337(330)}$ Tryptophan-$W_{86(84)}$[b] Phenylalanine-$F_{449(442)}$[b] Glutamate-$E_{202(199)}$[b]
3. Rim of the gorge (peripheral site)	Little direct influence; only allosteric	Fasciculin Propidium	AChE AChE	Tyrosine-$Y_{72(70)}$ Tyrosine-$Y_{124(121)}$
4. Rim of the gorge and choline subsite	Similar to 2 and 3	BW284c51	AChE	Tryptophan-$W_{286(279)}$[b] Aspartate-$D_{74(72)}$[b] Residues in domains 2 and 3

[a] The first subscript corresponds to the numbering system for mammalian AChE; the number in parentheses is the Torpedo numbering system.
[b] Conserved residues in AChE and BuChE.

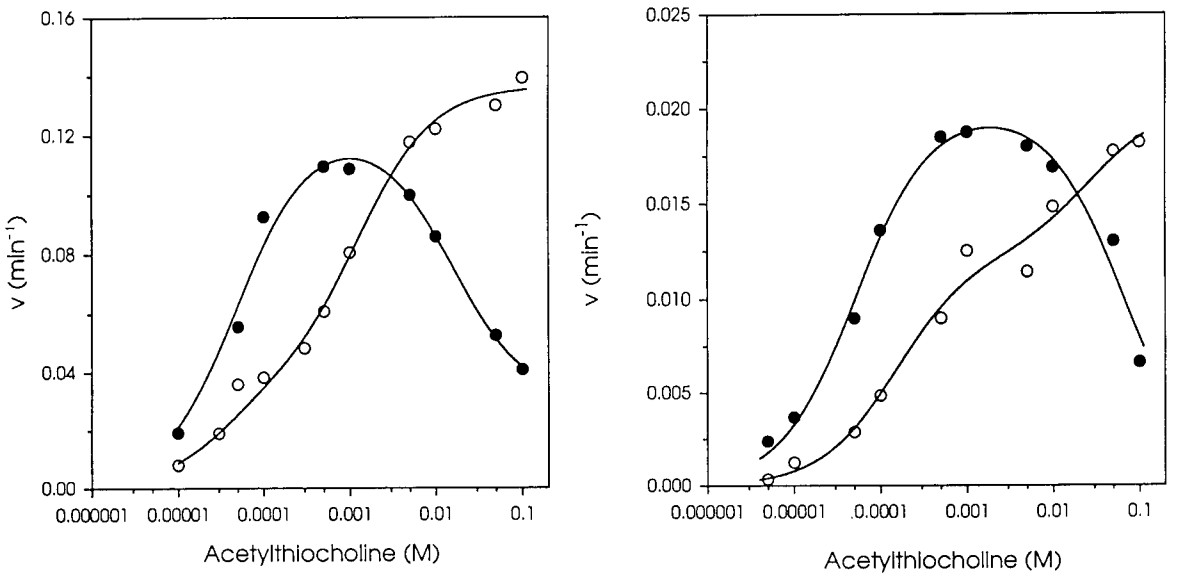


Fig. 1. Rates of acetylthiocholine hydrolysis as a function of substrate concentration. Left hand panel: Wild-type Mouse AChE (●); Wild-type Mouse BuChE (○). Right hand panel: Mutant $F_{295}L$ AChE (●); Mutant $F_{297}I$ AChE (○). The catalytic constants obtained from triplicate kinetic analyses and enzyme titrations are detailed in Table 1. Data are from Refs. [9,10].

as the alkylphosphonates, we can gain additional information on the specificity of enzyme acylation. Deacylation of the phosphonyl enzyme conjugates occurs slowly and may be studied as a separate step.

Berman and colleagues have found that a series of alkoxyl methyl phosphonylthiocholines show a 200- to 300-fold preference with the S_p-isomer over the R_p-isomer for *Torpedo* AChE [11,12]. A similar preference is seen with the mammalian enzyme and, through site-specific mutagenesis, we are able to define the structural determinants for enantiomeric specificity. It appears that the phosphonyl oxygen must be placed in the oxyanion hole and the leaving group directed out of the gorge to maximize acylation rates. A knowledge of the absolute stereochemistry of the phosphonates then reveals that the S_p-enantiomer positions its bulky alkoxyl group toward the choline binding site, while the small methyl group occupies the more space restrictive acyl pocket. On the other hand, the same constraints on the positioning of the phosphonyl oxygen and the leaving group require the bulky alkoxy group of the R_p-enantiomer to be oriented towards the acyl pocket where steric limitations do not allow an optimal fit. This orientation is consistent with the kinetic differences in S_p- and R_p-acylation, for as we enlarge the acyl pocket, we find that the R_p-isomer becomes a more efficient acylating agent [10] (Table 2). Other studies which compare cationic thiocholine and neutral thioalkyl leaving groups of these phosphonates are also consistent with this interpretation for orientation (N.A. Hosea, unpublished observations). Fig. 2 shows the orientations of S_p- and R_p-cycloheptyl methylphosphonylthiocholine when reversibly bound to AChE and $F_{297}I$ AChE, respectively.

3. The choline binding site in the active center

Tricyclic phenothiazine and acridine analogues are effective inhibitors and appear to associate with the choline subsite, located within the active center of the cholinesterases [5,9]. This region is also bounded by several aromatic side chains. While most tricyclic compounds do not exhibit a preference for AChE over BuChE, ethopropazine is a specific BuChE inhibitor with a 1800-fold preference over AChE (Table 3) [9].

Table 2
Influence of acyl pocket dimensions on substrate specificity

	Acetylthiocholine				Butyrylthiocholine				Cycloheptyl methyl phosphonothiocholine	
	K_m	k_{cat}	K_{ss}	b	K_m	k_{cat}	K_{ss}	b	k_a-S_p	k_a-R_p
AChE	46	140×10^3	13	0.21	93	1.1×10^3	7.1	0.48	1.8×10^8	0.008×10^8
BuChE	23	40×10^3	1.0	3.9	55	37×10^3	1.7	2.0	4.7×10^8	0.067×10^8
$F_{295}L$	52	44×10^3	67	<0.2	10	12×10^3	23	0.40	0.66×10^8	0.087×10^8
$F_{297}I$	170	15×10^3	43	1.8	82	60×10^3	2.4	1.7	0.16×10^8	0.62×10^8

K_m (μM), k_{cat} (min^{-1}), K_{ss} (mM), k_a (M^{-1} min^{-1}) and b (dimensionless) were determined as described in Refs. [10,14].

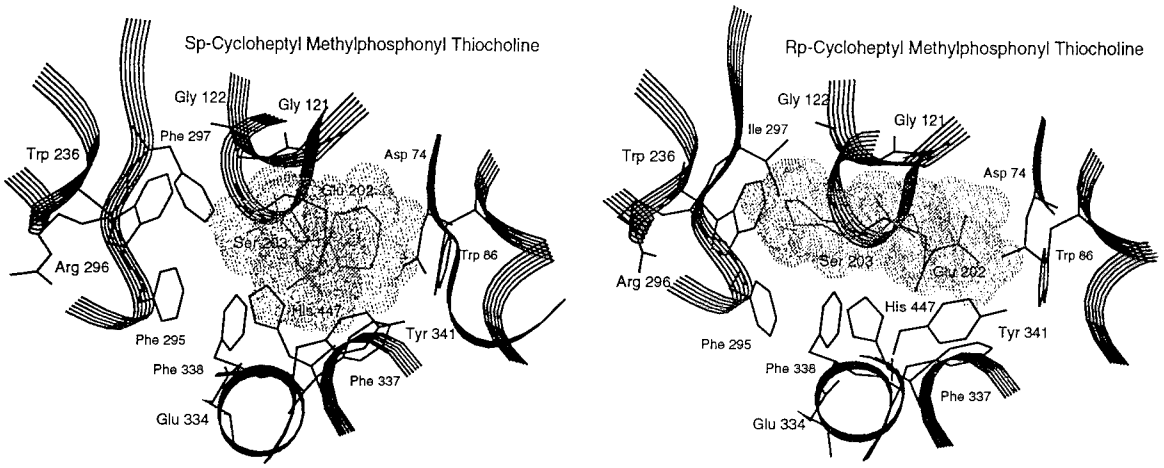


Fig. 2. Proposed orientations of R_p- and S_p-cycloheptylmethyl-phosphonylthiocholine in the active center of mammalian AChE. The model was developed from the coordinates of *Torpedo california* AChE [5]. Shown are the side chains of several of the catalytically (S_{203}, H_{447} and E_{334}) and structurally (F_{295}, F_{297}, W_{86}, F_{337}, G_{121}, G_{122}, E_{202}, D_{74}) important residues in the active center. In both enantiomers the phosphonyl oxygen is directed back towards the plane of the paper forming hydrogen bonds with the amide backbone hydrogens of G_{121}, G_{122} and A_{204}. The thiocholine leaving group is directed out of the gorge toward the reader. The S_p-enantiomer is docked with the wild-type sequence and the R_p-enantiomer docked with the $F_{297}I$ mutant.

Since BuChE and AChE differ by having alanine versus tyrosine or phenylalanine at the 337 position, the basis for this specificity might arise from steric occlusion between the tyrosine or phenylalanine side chain at position 337 on AChE and the exocylic amine containing side chain on ethopropazine. Substitution of alanine for tyrosine at this position in AChE confers high affinity for ethopropazine binding to AChE substituted at a single position. In fact, the change in energy ($\Delta\Delta G$) is remarkably similar to the AChE-BuChE difference (Table 3). Docking of

Table 3
Specificity at the choline binding site[a]

Ethopropazine	K_d (nM)	$K_{d,M}/K_{d,WT}$	$\Delta\Delta G$ (kcal)
Mouse BuChE Wild Type	61	1.0	0
Mouse AChE Wild Type	110 000	1800	4.5
$Y_{337}A$ AChE Mutant	41	2700	4.7

[a] Mammalian AChE has tyrosine (Y) at the 337 position, while BuChE has alanine. $\Delta\Delta G$ was calculated from the ratio of dissociation constants.

the ethopropazine molecule in a BuChE template and energy minimization show that the diethylaminoisopropyl moiety occupies the same region as would the phenyl ring at position 337 in AChE [9]. Hence, the specificity difference for this widely used inhibitor results from steric hindrance by a single aromatic side chain.

4. Fasciculin and the peripheral site on AChE

A peripheral site of inhibitor binding to AChE was defined many years ago on the basis of steady-state kinetics [13,14] and by direct titration with propidium [14]. This site is also believed to be the locus through which substrate inhibition is allosterically transmitted to the active center [15]. A family of peptides of 6750 Da found in the venom of mambas, termed the fasciculins, also appear to bind to the peripheral site on AChE with high affinity, but they are ineffective inhibitors of BuChE. Modification of residues comprising the peripheral site at the rim of the gorge and the active center deep within the gorge shows that only those residues at the rim of the gorge appreciably affect fasciculin binding [16]. In fact, substitutions of R for W_{286}, N for Y_{72} and Q for Y_{124} result in a large reduction of fasciculin affinity ($K_d = 2.3$ pM vs. 220 μM). These substitutions reflect BuChE-AChE sequence differences. This increase in K_d is equivalent to 11 kcal of free energy and is exactly the same as the energy difference seen between AChE and BuChE (Table 4).

By contrast, modification of a tryptophan at the base of the gorge, W_{86}, results in unusual and intriguing kinetic behavior for the fasciculin complex [17]. In the uncomplexed enzyme, the substitutions of tyrosine, phenylalanine and alanine for the tryptophan result in progressive reductions in catalytic efficiency, k_{cat}/K_m. However, the fractional inhibition of ACh catalysis by fasciculin is proportionally decreased in the respective fasciculin mutant-AChE complexes. Hence, in no case does saturating fasciculin result in 100% inhibition of catalysis, but fractional inhibition is greatest with the more active mutants. In fact, the fasciculin complexes of the various enzymes mutated at position 86 have relatively similar catalytic efficiencies.

Table 4
Dissociation constants for fasciculin with wild-type and mutant acetylcholinesterases and butyrylcholinesterase

Enzyme	K_i (pM)
AChE (wild type)	2.3 ± 0.7
$F_{295}L$	16 ± 8
$F_{297}I^{\beta}$	57 ± 15
$F_{297}Y$	7.9 ± 1.7
$F_{338}G$	7.9 ± 1.7
$Y_{337}A$	4.2 ± 1.7
$D_{74}N$	43 ± 7
$Y_{124}Q$	248 ± 57
$Y_{72}N$	7800 ± 900
$W_{286}R$	2 100 000 ± 600 000
$Y_{72}N,Y_{124}Q$	72 000 ± 19 000
$Y_{124}Q,W_{286}R$	8 500 000 ± 3 100 000
$Y_{72}N,W_{286}R$	170 000 000 ± 66 000 000
$Y_{72}N,Y_{124}Q,W_{286}R$	235 000 000 ± 60 000 000
BuChE (wild type)	210 000 000 ± 98 000 000

If a neutral and less efficiently catalyzed substrate such as *p*-nitrophenylacetate is used instead of acetylthiocholine, fractional inhibition by fasciculin is diminished. Moreover, fasciculin is a less effective inhibitor of *p*-nitrophenylacetate hydrolysis by the mutant enzymes, and for the fasciculin-W_{86}A AChE complex is a more efficient catalyst than is W_{86}A AChE alone. Hence, for this mutant enzyme of diminished catalytic efficiency, fasciculin behaves as an activator rather than an inhibitor [17].

These data not only reveal the surface of fasciculin binding on the enzyme, but also confirm previous results with diisopropylfluorophosphate labeling in which the gorge remains open, or partially open, in the fasciculin complex [18]. A more detailed kinetic analysis shows that fasciculin may gate entry to the gorge for those substrates whose catalysis is limited or near-limited by diffusion. In the case of poorer substrates, such as *p*-nitrophenylacetate, whose catalysis is limited by substrate orientation or a similar unimolecular isomerization step [6], substrate entry is not rate limiting nor does it become rate limiting in the presence of fasciculin. Hence, the primary influence of fasciculin for these substrates is to induce a conformational change which affects chemical reactivity and orientation of the bound substrate. In most cases this diminishes catalytic efficiency, except for the W_{86}A

mutation where catalysis of *p*-nitrophenylacetate is enhanced slightly.

The binding of propidium at a peripheral site has also been shown to affect the conformation of the active center and catalytic efficiency of AChE [3]. Some of the residues involved in allosteric control by propidium have also been defined [19].

A knowledge of the structures of fasciculin and the cholinesterases enables one through mutagenesis to analyze which side chains in peptide and protein are energetically coupled in the formation of the complex. This approach should distinguish which residues in fasciculin contribute to formation of the surface of interaction, to alterations in kinetics through steric constraints or charge repulsion, and to inducing conformational changes in AChE. Hence, the wealth of natural and synthetic inhibitors of the cholinesterases will continue to yield important molecular details on the function of this family of enzymes.

Acknowledgements

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Neuropathy target esterase (NTE) and organophosphorus-induced delayed polyneuropathy (OPIDP): recent advances

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Abstract

The identification of neuropathy target esterase (NTE) as the site for initiation of organophosphorus-induced delayed polyneuropathy (OPIDP) has led to informative acute and chronic neurotoxicity tests (adopted by OECD and EPA), to structure/activity and in vitro/in vivo predictions, and to a sound basis for extrapolations to man. Purification of the sodium dodecyl sulphate (SDS)-denatured 155-kDa sub-unit of NTE has enabled partial sequencing and molecular biological studies. A MAb to the chicken brain sub-unit and PAbs to synthetic peptides have been raised: preliminary experiments suggest that one is effective for immunohistochemistry of frozen tissue. cDNA libraries are being screened with synthetic oligonucleotides, polymerase chain reaction (PCR)-developed primers, and with Ab in order to obtain cloned NTE. Previous studies of NTE in vivo have not revealed its normal physiological function or the route from inhibition to degeneration of axons, but the current progress in molecular biology of NTE is applicable to study of the function of normal and organophosphorus (OP)-modified NTE in cultured neural cells.

Keywords: Neuropathy target esterase; Esterase purification; Organophosphorus neuropathy protection; Neuropathy promotion

1. Introduction

References to the early work summarised here are contained in Refs. [1–6].

Following the 'Ginger Jake' epidemic in the 1930s caused by the consumption of contaminated liquor, tri-*o*-cresyl phosphate (TOCP) was identified as the main aetiological agent, using the adult hen as an experimental model. In the 1950s a clinically similar poisoning episode occurred during pilot plant synthesis of a new candidate pesticide (mipafox; *N,N*/di-*iso*-propylamino-phosphorofluoridate) and the condition was reproduced in the chicken. Some other organophosphorus (OP) compounds such as di-*iso*propyl phosphorofluoridate (DFP) were shown to produce the disease but many were inactive; lesions were found in the long axons of the central and peripheral nervous system. A minimum delay of 10 days or so between the administration of active compounds and the appearance of clinical signs in chickens and

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Abbreviations: TOCP, tri-*o*-cresyl phosphate; OP, organophosphorus; DFP, di-*iso*propyl phosphorofluoridate; OPIDP, organophosphorus-induced delayed polyneuropathy; NTE, neuropathy target esterase; S9B, 1-(saligenin cyclic phospho)-9-biotinyldiaminononane.

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humans always occurred. With the testing of potential pesticides for veterinary use many species were shown over some years to be susceptible. The list now includes the cat, dog, horse, cow, sheep, pig, water buffalo, ferret and monkey but small laboratory rodents and most birds were not affected easily. In all species the clinical conditions and the distribution of the histopathological lesions in the central and peripheral nervous system were similar.

Up to the 1960s attempts to find the mechanism of organophosphorus-induced delayed polyneuropathy (OPIDP) were based on the 'guess and test' system, and the involvement of various cholinesterases and esterases in the nervous system was eliminated. In the late 1960s a more logical approach was adopted and an hypothesis was devised with the following components; (i) OPIDP being initiated by covalent organophosphorylation of a molecular target; (ii) this target would, almost certainly, be a protein having esterase activity; (iii) the esterase activity to be inhibited in vivo by covalent binding of neuropathic OP compounds (such as DFP, TOCP, mipafox, etc.) given at relevant doses and not inhibited by those compounds which do not cause the disease; (iv) substrate hydrolysis catalysed by this esterase in vitro would be inhibited by neuropathic compounds provided that in vivo they did not need to be bioactivated; (v) if bioactivation in vivo were necessary then (iv) would apply only to the bioactivated and proximal toxic agent; (vi) inhibition in vitro would not be caused by those which did not produce the disease such as tetraethyl pyrophosphate and paraoxon (diethyl-4-nitrophenyl phosphate). A target protein having 'correct' target properties was discovered and then shown to be an esterase which is now known as neuropathy target esterase (NTE): methods were developed for its assay both by phosphorylation with [^{32}P]DFP and by the hydrolysis of phenyl phenylacetate and, later, of phenyl valerate. For a large number of OP compounds the 'NTE hypothesis' was verified, and active compounds produced delayed neuropathy in chickens when NTE was at least 70–80% inhibited; no disease occurred with less or no inhibition.

2. NTE and toxicity tests

NTE has proved to be an excellent tool for assessment of neuropathic potential of OP compounds. This is expounded at length in Ref. [5]. Briefly:

(i) NTE tests are now an essential component of regulatory tests recommended both by EPA and OECD. A great advantage is that each and every degree of inhibition that is found in autopsy samples provides some guidance – the neuropathic effect of a given dose can be predicted to be positive, marginal or thoroughly negative. The recommended tests allow that toxicokinetics must be considered, and assays should ensure that the peak effect is identified: this may not occur 1 day after dosing, as envisaged in early studies.

(ii) Effects of repeated administration (chronic or sub-chronic toxicity) also can be evaluated, provided that assays are performed at suitable times (NOT weeks after the final dose!).

(iii) Prediction of neuropathic potential in vivo has been made possible by comparison of the relationships of chemical structure of OP compounds to their activity in vitro against NTE and acetylcholinesterase.

(iv) The comparisons in (iii) above which used hen brain to predict results in avian tests have been successfully extended to studies with human autopsy samples: they have shown that the hen appears to be an excellent model for human. Such tests did detect a few compounds which might confidently be thought to be either somewhat more or slightly less neuropathic in humans than in hens and these conclusions seem to have been valid in so far as human intoxications with such compounds have been observed. It would be prudent to assume that the threshold of inhibition in humans required to precipitate clinical neuropathy may be less than the 70–90% value for hens, but no convincing evidence has been reported.

3. The nature and function of NTE

3.1. The enzymic character of NTE

NTE behaves as a typical 'B' (OP-sensitive)

esterase rapidly hydrolysing the optimum artificial substrate (phenyl valerate) but no physiological substrate has been identified. Many characteristics (distribution, sensitivity to pH, to non-OP inhibitors, etc.) are summarised by Johnson [4,6]. Extensive structure/activity studies for both substrates and inhibitors in vitro were reported in 1977 and 1988 (see Ref. [6]) and also recently from the laboratories of Casida and Hammock [7,8]. A few substrates are hydrolysed more rapidly than phenyl valerate but their specificity for NTE is poorer so that the precision of the differential assay becomes less, particularly when some fraction of the NTE has been inhibited [6,8].

It has been shown recently that NTE is present in even higher concentration in bovine adrenal medulla than in neural tissue [9]. Good concentrations of NTE have also been found in conveniently cultured tissues such as bovine chromaffin cells (private communication from Dr. Vilanova) and in some human neuroblastoma cells [10].

Isolation of NTE from its site of firm attachment to neural membranes (the intracellular origin is uncertain) has progressed only recently. Rüffer-Turner et al. [11] prelabelled hen brain microsomes with [^{3}H]DFP and were able by conventional procedures to isolate the labelled NTE sub-unit (mol. wt. = ca. 155 kDa) free of other labelled polypeptides but probably only about 2% pure in terms of other protein. Digestion and separation studies produced labelled fragments centred around 30 and 16 kDa. Further digestion studies obtained a small peptide containing the typical consensus sequence for a serine ('B') esterase, namely Gly-Glu-Dehydro-ala [derived from DFP-labelled Ser]-XXX-Gly [12]. Similar peptides, this time labelled with biotin, have been obtained from what appears to be pure 155 kDa polypeptide isolated in our laboratory [13] by a simple procedure using a bidentate affinity agent, S9B (1-(saligenin cyclic phospho)-9-biotinyldiaminononane): the peptide is, of course, catalytically inactive having been both OP-inhibited and then processed in sodium dodecyl sulphate (SDS). We have heard rumours of further progress towards obtaining pure catalytically active NTE building on the beginnings of Thomas et al. [14].

3.2. Protein and molecular biology studies of NTE

As indicated above, a number of fragments of tritium or biotin-labelled NTE have been examined. Based on sequences obtained, synthetic oligonucleotides have been synthesised and cDNA libraries of brain from hen and other species are being screened with such, with polymerase chain reaction (PCR)-developed primers and with antibody (see below). We can only report that cloning work continues. We have raised a monoclonal antibody to SDS-denatured 155-kDa polypeptide from hen brain and a polyclonal Ab to synthetic fragments of pig brain NTE (details to be published elsewhere). These Abs are effective agents for screening in vitro and preliminary experiments suggest that one is suitable for identifying NTE in histochemical studies of isolated tissue.

3.3. NTE has some aspects of a receptor

It was shown 20–25 years ago that initiation of OPIDP depended not on the loss of catalytic activity of NTE in vivo but on a subtle change in the NTE molecule itself or in its environment brought about as a result of the covalent binding of the initiating agents [2,4–6]. Phosphate and phosphonate inhibitors of NTE tended to undergo a rapid bond-cleavage reaction ('ageing') after attachment to the active site: this involved generation of a negatively charged residue at the active site plus intramolecular transfer of the cleaved group to an adjacent site (nature unknown). This event subsequent to inhibition of NTE appeared to be essential since predosing hens with non-'ageable' inhibitors of NTE (appropriately structured carbamates, sulphonyl fluorides or phosphinates) totally prevented neuropathic effects of challenge doses of neuropathic OPs (DFP, etc.) without preventing acute (anticholinesterase) toxicity. Johnson [15] discussed the concept of NTE as a fortuitous receptor and this has been developed by Lotti [16,17]. It had been discovered in several laboratories [18–20] that post-dosing any of those

prophylactic compounds into hens which had received a low 'priming' (i.e. not in itself sufficient) dose of DFP led to expression of a full clinical neuropathy. Lotti et al. [17] then arranged doses of a few OP compounds and one carbamate to achieve similar degrees of inhibition of NTE and showed that post-dosing with phenylmethanesulfonyl fluoride produced differing severities of clinical response. The experiments are complex, the controls are difficult, and the differences modest, but the case that the initiatory effect differs according to the nature of the covalent agonist appears to be sound. These findings add to the conviction that initiation proceeds from events in the region of the active site serine of NTE rather than at the site (dubbed 'Z') receiving the cleaved group. Further powerful reasons are that several phosphoramidates have been shown in our laboratory to initiate OPIDP by inhibition without apparent 'ageing/group-transfer (summarised in [21]) and it has been pointed out by Richardson [22] that phosphoramidated NTEs could undergo electronic shifts in the region around the phosphorus atom at physiological pH to generate negatively charged structures typical of Westheimer Acids. Such changes do not involve cleavage of any alkyl group (so no transfer to site 'Z') and would generate a phosphyl-enzyme bond fairly but not intractably resistant to the reactivating power of standard nucleophilic reactivating agents (as we had observed [23]).

4. NTE in vivo

NTE is widespread in tissues (listed in [5,6,16]). Apart from the adrenal medulla noted above (Section 3.1) the brain has highest concentrations while the tissues principally subject to axonal degeneration (spinal cord and peripheral nerve) have less. The presence of substantial amounts of NTE in spleen and lymphocytes has raised the question of an immunological significance for the enzyme, but no direct evidence exists and attempts to implicate the immune system in OPIDP have failed [2,6]. No profound differences have been found between NTE from different species or tissues [6] but the existence of low concentrations of a soluble form in both brain (where it is a very minor component) and sciatic nerve (ca. 50% of the total amount in that tissue) is interesting, and some modest differences between soluble and particulate forms have been reported [24].

Although prolonged inhibition of NTE without ageing does not cause neuropathy or other obvious adverse effects this does not necessarily mean that the esteratic activity of NTE is not part of a normal physiological process but only that any such process is not vital or rate-limiting. It may be that the system can survive long periods with only a small proportion of its esteratic capacity or that the substrate can be channelled through another route. However no obvious naturally occurring analogues of the best NTE substrates appear to be candidates for a true physiological substrate. The possibility that interactions of OP compounds with the active site of NTE mimic the control processes mediated by protein kinases seems to have no substance [6]. Turnover studies and measurements of transport of NTE in axons have been reported (summarised in [6]) but none have indicated anything unusual or given a hint as to function of this enzyme/receptor.

5. Conclusion

For toxicologists NTE has become firmly established as an assayable target. A disturbance of the charge distribution at the active site of inhibited NTE appears to be the initiating event in OPIDP. The normal function of NTE remains a mystery, but its recent isolation and current progress in the immunochemistry and molecular biology of NTE and the identification of NTE in easily cultured cells offers hope that this function can be investigated in the near future.

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Receptor-mediated toxicity

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Abstract

Toxic agents, particularly those that exert their actions with a great deal of specificity, sometimes act via receptors to which they bind with high affinity. Some examples of soluble intracellular receptors, which are important in mediating toxic responses, are discussed in this paper. The first example is the glucocorticoid receptor, which can act as a model for other receptors but is also involved in mediating toxicity associated effects such as apoptosis of lymphocytes as well as neuronal degeneration as a response to stress. Other examples discussed are the peroxisome proliferator activated receptor (PPAR) which is associated with hepatocarcinogenesis in rodents, and the dioxin receptor which is involved in a whole range of toxic effects. The final example given is an interesting mechanism for tissue specific accumulation of a metabolite of polychlorinated biphenyls.

Keywords: Receptor-mediated toxicity; Glucocorticoid receptor; PPAR; Dioxin receptor; Polychlorinated biphenyls

The glucocorticoid receptor belongs to the nuclear receptor supergene family which also encompasses the progesterone receptor, the mineralocorticoid receptor, the estrogen receptor, the androgen receptor, the retinoic acid receptor, the thyroid hormone receptor, the vitamin D_3 receptor as well as many so-called orphan receptors which lack a known ligand. All these receptors share the same principal three-domain structure with a central DNA-binding domain, a C-terminal ligand binding domain and an N-terminal domain with highly variable length. The DNA-binding domain has a two zinc finger structure where each zinc atom coordinates four cysteines. The three-dimensional structure of the DNA-binding domain of the glucocorticoid receptor was first reported by our group in collaboration with Dr Robert Kaptein, Utrecht, Holland [1]. NMR analysis of recombinant protein revealed a structure with two α-helices perpendicular to one another where the recognition helix enters the major groove on DNA and binds to specific bases constituting the glucocorticoid responsive element. The DNA-binding domain interacts with DNA as a dimer and the overall structure of the DNA-binding domain is identical for all members of the nuclear receptor supergene family.

Chambon's group described the three-dimensional structure of the ligand binding domain of the RXR receptor [2]. Very recently, Baxter reported on the three-dimensional structure of the thyroid hormone receptor ligand binding domain [3]. In contrast to Chambon, Baxter crystallized the ligand binding domain with bound ligand, enabling molecular modeling studies for development of new agonists and antagonists to thyroid hormone. Again, the overall structure of the ligand binding domain appears to be quite similar between RXR and

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thyroid hormone receptor, and it is expected that this is true also for the other members of the nuclear receptor supergene family.

The N-terminal part of the glucocorticoid receptor contains the major transactivation domain which is responsible for regulation of gene expression of target genes for glucocorticoids. A great deal of interest is currently focussed on which proteins of the basal transcriptional machinery are contacted by the transactivation domains of various receptors. We are also addressing this problem and our data indicate that several proteins are contacted by the major transactivation domain, τ_1, of the glucocorticoid receptor. Based on experiments with yeast we have defined a 58 amino acid long τ_1 core which is responsible for most of the transactivation activity of the major transactivation domain [4]. The τ_1 core contains many acidic amino acids and hydrophobic amino acids. Phosphorylation, however, does not seem to play a role in receptor activation of target genes based on experiments involving mutated amino acid residues and, furthermore, the acidic amino acids also appear to be of relatively little significance in regulation of gene expression. Finally, it would seem that the hydrophobic amino acids are of prime importance [5].

We have expressed the τ_1 core in bacteria and studied its overall structure with the help of circular dichroism. Somewhat disappointingly, neither the τ_1 core nor the full-length τ_1 showed any two-dimensional structure. However, it was argued that the normal environment for the τ_1 core when contacting components of the basal transcriptional machinery might be quite hydrophobic and, thus, trifluoroethanol (TFA) was added to the protein solution in order to mimic the hydrophobic environment of the protein under natural conditions. Addition of TFA had a dramatic effect on the protein, which following such treatment showed induction of α-helical structure. NMR analysis of the TFA treated protein revealed the existence of three α-helices. With the help of helix breaking proline substitutions, it could be shown that the integrity of at least two of the α-helices was necessary for transactivation to occur [6]. We are currently studying these issues further, but it would seem that 'hydrophobic patches' on the α-helices are especially important, and it might be suggested that these patches contact hydrophobic proteins in the basal transcriptional machinery and associated pool of coactivator proteins [5].

A very important aspect with reference to nuclear receptors is cross-talk, whereby the receptors interact with other signal transduction pathways. One such example will be given. In the NFκB system, the transcription factors p65 (also called Rel A) and p50 form a heterodimer which interacts with κB-elements on regulated genes. Normally, the heterodimer is unable to bind to DNA due to an inhibitor, called IκB protein. Activation of the NFκB-system is caused by a large number of stimuli, e.g. factors liberated during inflammation, and it would seem that the common mechanism whereby all these stimuli result in activation of the NFκB-system is the release of H_2O_2 which leads to phosphorylation and breakdown of IκB, thus releasing the p65/p50 heterodimer. Interestingly, as we have recently reported, the glucocorticoid receptor can shut down the NFκB signalling by interacting with the p65 component of the complex [7]. The inhibition is reciprocal, which means that p65 can also inhibit glucocorticoid receptor activity, simply by binding to it. This mechanism is probably important in explaining at least part of the negative effects of glucocorticoids on the inflammatory response. Cross-talk between steroid receptors and other signal transduction pathways is probably extremely common and needs to be investigated in detail for a full understanding of the spectrum of biological activities of the receptors.

The second theme relates to the orphan receptors mentioned above. More specifically, I will discuss the peroxisome proliferator activated receptor or PPAR which some years ago was shown to mediate the effects of peroxisome proliferators [8]. The peroxisome proliferator activated receptor has already qualified as a subfamily of receptors in its own right. Several species seem to contain at least three isoforms, α, γ and δ (also called β or NUC1). These isoforms have a different distribution in various tissues. We reasoned that it is most likely that PPAR has a physiological ligand and, knowing the impor-

tant role of PPAR in regulating activities of genes involved in fat metabolism, it was not inconceivable that the ligand might be of lipid nature. We developed an assay for the natural ligand of PPARα by fusing the ligand binding domain of PPARα to the N-terminal and DNA-binding domains of the glucocorticoid receptor under the control of a metallothionein promotor. The reporter gene consisted of MMTV-LTR containing several GREs placed in front of alkaline phosphatase which encodes a product that is secreted from the cells (CHO cells) and easily assayable in the medium using a colorimetric reaction. As a source for activating substances we used human pregnancy plasma which was fractionated over several chromatographic columns. With this approach we could show that PPAR activation capacity was invariably associated with the fatty acid fraction [9]. The notion that fatty acids are endogenous regulators of PPAR was further substantiated by adding a range of reference fatty acids to the cells; the ligand specificity turned out to be quite broad (from C7 and upwards). Unsaturated fatty acids were about three times as active as saturated fatty acids.

In order to monitor the activation response, it was necessary to add the fatty acids at concentrations of about 100 μM. This was of some concern to us since nuclear receptor ligands normally interact with their receptors at nM concentrations. In order to study direct binding of fatty acids to the PPAR, a photoaffinity label (para-azidophenoxynonanoic acid) was used. By titrating the concentration of this photoaffinity label with the help of recombinant fatty acid binding protein or delipidated bovine serum albumin, it could be shown that recombinant PPARα overexpressed in baculovirus infected insect cells was radioactively labeled at fatty acid concentrations in the nM range [10]. Based on these experiments it can be concluded that free fatty acids are the most likely ligands for PPAR, and this conclusion is also based on several experiments where we have failed to block the fatty acid activation of PPAR by adding inhibitors of fatty acid metabolism [11].

In order for PPAR to interact with DNA it is necessary for PPAR to form a heterodimer with retinoid X receptor or RXR, another member of the nuclear receptor supergene family. Since RXR is also a partner for the thyroid hormone receptor and the vitamin D_3-receptor as well as the retinoic acid receptor, the possibility exists that under certain conditions RXR may exist in limiting concentrations and that the various involved signal transduction pathways might cross-talk to one another via RXR. In this way, activation of PPAR by for instance peroxisome proliferators might have consequences for signaling also in other systems. In order to investigate more systematically which other proteins that PPARα might contact, we are currently using the two hybrid system in yeast. We have already identified several genes whose products seem to interact specifically with PPAR ligand binding domain [12]. In particular, a kinase we have identified in this way is of potential interest in view of the possibility that phosphorylation events might play a role in the activation of PPAR, as has been shown in the case of, for instance, the progesterone receptor.

Finally, in collaboration with Dr Chris Corton at Chemical Industry Institute of Toxicology (CIIT) we currently use the differential display technique in order to identify new target genes for PPAR. With this technique we have recently identified a 17β-hydroxysteroid dehydrogenase type IV which converts 17β-estradiol to estrone [13]. Northern blot analysis confirms that this 17β-estradiol oxidase is induced about four- to five-fold following treatment with peroxisome proliferators, and these results might offer an explanation for the hypoestrogenic effect sometimes seen following administration of peroxisome proliferators.

The dioxin receptor is involved in a multitude of toxic responses, and one of our specific interests with reference to this receptor is the nature of its physiological ligand, if it exists. The dioxin receptor induces a cascade of genes following activation, for instance the cytochrome P450 IA1 and IA2 which are both involved in metabolic activation of promutagens. Somewhat surprisingly to many workers in the field, it was shown some years ago that the dioxin receptor does not belong to the nuclear receptor supergene family but instead belongs to the so-called basic helix-

loop-helix family of transcription factors. These are known to play important roles in e.g. embryogenesis, and muscle development is regulated by members of this transcription factor class. The dioxin receptor system has been shown to consist of two components, the ligand binding dioxin receptor as well as its partner protein Arnt. Both belong to the basic helix-loop-helix class of transcription factors. In the non-liganded state, the dioxin receptor is complexed with heat shock protein 90 (hsp 90) which serves a dual function. It seems to prevent the dioxin receptor from interacting with DNA and at the same time it seems to be necessary to uphold the proper conformation of the ligand binding domain. Following ligand activation, hsp 90 dissociates and the dioxin receptor is free to heterodimerise with Arnt via the basic helix-loop-helix domain, and DNA binding to xenobiotic-responsive elements takes place.

In order to functionally dissect the dioxin receptor, we have used a chimera approach similar to that described above concerning the PPAR. Thus the dioxin receptor from which the N-terminal basic helix-loop-helix domain was deleted was fused to the N-terminal domain and the DNA-binding domain of the glucocorticoid receptor. In this way a receptor chimera was created which turned out to be activated by dioxin and then bind to glucocorticoid responsive elements [14]. Using various deletions, it could be shown that ligand binding takes place over the PAS B domain in the dioxin receptor. This forms part of the PAS domain which is common to per, Arnt, sim and the dioxin receptor, hence the name PAS domain. Per and sim are basic helix-loop-helix transcription factors which have been described in Drosophila. The PAS domain contains two repetitive sequences, A and B. Also, hsp 90 interacts with the PAS B domain, in line with its conformation stabilizing effect on the ligand binding domain. A transactivating function was identified in the C-terminal part of the dioxin receptor. In the same way the Arnt partner factor was dissected functionally. Arnt was shown not to interact with hsp 90 and does not interact with tested ligands. As mentioned above, Arnt also has a PAS domain and, similarly to the dioxin receptor, it carries a transactivating function in the C-terminal part of the protein. The absolute necessity of hsp 90 for signalling via the dioxin receptor pathway was shown using yeast cells devoid of hsp 90 but where the hsp 90 gene had been reintroduced under a galactose inducible promotor. Thus, by varying the galactose concentration in the growth medium of yeast it was possible to conveniently modulate hsp 90 concentration in the yeast cells. Dioxin receptor function was studied using the above-mentioned chimera and it could be clearly shown that no activation of the dioxin receptor/glucocorticoid receptor chimera occurred on a GRE-driven reporter gene in the absence of hsp 90, whereas cultivation of yeast cells in the presence of galactose leads to reconstitution of the dioxin signaling [15]. Interestingly, comparison between the mouse and human dioxin receptors in this system showed that the human receptor was one order of magnitude less sensitive than the mouse receptor with reference to activation by β-naphtoflavone, a fact of some interest from the regulatory point of view.

Concerning ligands of the dioxin receptor, we were interested to see whether heterocyclic amines formed during frying of food might possibly interact with the dioxin receptor since some of the heterocyclic amines have planar structures reminiscent of dioxin, and we wanted to investigate whether these particular mutagens could stimulate their own metabolism by activating the dioxin receptor. This was indeed shown to be the case, first in vitro where the dioxin receptor, using gel shift assays, was activated to the DNA-binding state by several of the tested heterocyclic amines [16]. Also in cells with functional dioxin receptor system and XRE-driven reporter genes, heterocyclic amines were shown to activate the reporter gene, albeit at relatively high concentrations [17].

During the 1960s, Lee Wattenberg reported that consumption of brussels sprouts leads to the induction of cytochrome P450 activity in the gut [18]. He ascribed this effect of brussels sprouts to the dietary component indole-3-carbinol which, however, was devoid of dioxin receptor-binding activity. We found evidence in the literature that

indole-3-carbinol under certain conditions could be converted to indolocarbazole, and decided to test whether this particular metabolite of indole-3-carbinol could serve as a ligand to the dioxin receptor. This was indeed the case, and in particular methylsubstituted indolocarbazoles were shown to bind to the dioxin receptor with a K_d around 1 nM [19,20]. Thus, indolocarbazoles interact with the dioxin receptor with the same affinity as dioxin itself. Indeed, when gelshift analyses were carried out comparing activation of the mouse receptor and the human receptor, it could be shown that indolocarbazole was a more efficient activator of the human dioxin receptor than dioxin itself [21], again emphasizing the need to base regulatory decisions on human receptor results rather than on rodent receptor experiments.

In situ hybridization studies of developing rat embryos indicate that Arnt has a much more widespread occurrence than the dioxin receptor. On days 16 and 18 the latter receptor was mainly localized to the oral cavity and the gastrointestinal tract, whereas Arnt appeared to be almost ubiquitously expressed [22]. These results indicate that Arnt may serve to bind several partners other than the dioxin receptor, perhaps in the same way as RXR is a promiscuous partner among nuclear receptors. If this notion holds true, the possibility opens up that the dioxin receptor might cross-talk with several other signal transduction pathways via Arnt, phenomena that might be of considerable significance in explaining some of the toxic effects of dioxin.

Finally, an example will be given of a macromolecule that is not a receptor but serves to concentrate very specifically metabolites of a toxic agent in a specific tissue.

Several years ago we observed that administration of radioactive polychlorinated biphenyls (PCBs) to mice resulted in the accumulation of radioactivity in the lung. It could be shown that this radioactivity represented methylsulfone metabolites of PCB and, in view of the pronounced specificity of the tissue accumulation, we argued that the most likely explanation might be the existence of a tissue specific protein interacting with the particular PCB metabolite. In the following studies we used radioactivelly labeled 4,4′-methylsulfone-2,2′, 5,5′-tetrachlorobiphenyl (PCB methylsulfone). The lung protein was purified and turned out to be uteroglobin, a protein that has previously been studied primarily in the uterus [23]. The PCB methylsulfone mainly accumulated in the Clara cells and, using immunogold-EM with antibodies against uteroglobin, this protein could be shown to be localized in secretory vacuoles of the Clara cells. The protein is secreted from the Clara cells and then forms part of the lining of the bronchiolar epithelium. We have recently managed to solve the three-dimensional structure of uteroglobin with the bound PCB methylsulfone using NMR [24]. The generated structure shows clearly how the PCB methylsulfone is situated in the central ligand binding cavity of uteroglobin, with hydrogen bonding between a tyrosine residue and the oxygen atoms in the methylsulfone moiety. We do not yet know whether the interaction between uteroglobin and the PCB methylsulfone results in a deranged function of uteroglobin (its function has not yet been elucidated), but it seems quite clear that the reason for the pronounced lung specific accumulation of the PCB metabolite is the tight interaction between the toxic agent and uteroglobin. This is of potential interest in view of the toxic symptoms described in lung tissue following exposure of the organism to PCBs.

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Toxicology Letters

Role of keratinocyte-derived cytokines in chemical toxicity

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Abstract

Following appropriate stimulation, such as with tumor promoters, ultraviolet light or various chemical agents, keratinocytes synthesize and secrete cytokines which can mediate or participate in dermatotoxic responses such as inflammation, hyperkeratosis, hypersensitivity and skin cancer. We have determined the qualitative and quantitative cytokine response in primary human keratinocyte cultures following exposure to several non-sensitizing contact irritants, sensitizers and ulcerative agents as well as a skin carcinogen. The chemicals were also administered to mice to assess whether the dermatotoxic response correlated with the in vitro production of keratinocyte-derived cytokines. Due to the complex cellular interactions that occur in the skin, it was not possible to identify specific cytokine profiles for most of the classes of dermatotoxic agents studied. However, the non-sensitizing contact irritants produced relative increases in the synthesis and secretion of the proinflammatory cytokines, interleukin-1 and tumor necrosis factor-α, as well as the neutrophil chemotactic cytokine, interleukin-8 compared to the other chemical agents. While ulcerative compounds as well as irritants elicited neutrophils to the site of chemical application when applied to the mouse skin, time-dependent and chemical-specific patterns of inflammation were detected. Treatment of human keratinocyte cultures with arsenic, a human skin carcinogen, resulted in a unique cytokine profile characterized by induction of growth factors, including transforming growth factor-α and granulocyte-macrophage colony stimulating factor. Treatment of v-Ha-*ras* transgenic mice, an animal model for skin cancer, with arsenic caused an increase in the number of papillomas as well as overexpression of these growth factors suggesting that they participate in arsenic-induced skin papilloma development. These studies indicate a diverse role exists for keratinocyte-derived cytokines in dermatotoxic actions.

Keywords: Inflammatory cytokines; Dermatotoxicity; Skin immunity; Keratinocyte-derived cytokines

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1. Introduction

Mammalian skin is a complex organ that contains many immune components. The term skin-associated lymphoid tissue (SALT) was coined to include the Langerhans cells with their antigen-presenting properties, the cytokine-producing keratinocytes, homing T lymphocytes, vascular endothelial cells, and the draining lymph nodes [1]. The cells of the skin can respond immunologically to environmental agents by the induction of allergic contact dermatitis, irritant contact dermatitis, antigen-specific systemic immunosuppression and hyperkeratosis. Although the primary function of keratinocytes is to provide structural integrity and barrier function of the epidermis, recent studies have demonstrated the importance of keratinocytes in the initiation and perpetuation of these dermatotoxic responses through the elaboration and release of cytokines and growth factors. For example, various environmental stimuli, such as tumor promoters, ultraviolet light and chemical agents can induce epidermal keratinocytes to release inflammatory cytokines (e.g., interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α)), chemotactic cytokines (e.g., IL-8), and growth promoting cytokines (e.g., granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor-α (TGF-α)) [2,3].

Since a broad spectrum of dermatotoxic agents exists, considerable efforts have been made to develop predictive tests that assess their potential toxicity and more recently, immunotoxicity. Many laboratory animal species have been used in skin irritancy testing but the most common has been the rabbit, where the skin test developed by Draize et al. [4] has received considerable attention. More recently, efforts have focused on various in vitro tests using reconstituted skin models to characterize dermatotoxic responses. Such tests have been used as 'prescreens', which if positive, may preclude the need for animal tests. Our studies have focused on attempts to employ cytokine synthesis and secretion from human primary keratinocyte cultures as a predictive model for the potential in vivo activity of dermatotoxicants. Details of these findings and methodology have been published elsewhere [5,6].

2. Materials and methods

2.1. Test chemicals

Benzalkonium chloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium arsenite, dinitrochlorobenzene (DNCB), phorbol acetate (TPA) and oxazolone were obtained from Sigma Chemical Co. (St. Louis, MO). Phenol and sodium lauryl sulfate (SLS) were obtained from J.T. Baker (Phillipsburg, NJ) and Schwartz/Mann Biotech (Cleveland, OH), respectively, while chromium oxide (CrO_3) was provided by Research Triangle Institute (Research Triangle Park, NC).

2.2. Cell culture and cytokine secretion

Cytokine secretion was quantitated from cultures of normal human epidermal keratinocytes (Clonetics, San Diego, CA) grown in keratinocyte growth medium (KGM; 5). When the cells were 55–65% confluent, the medium was changed to KGM without hydrocortisone or for arsenic studies also without bovine pituitary extract (a source of TGF-α). The cells were allowed to grow for an additional 24 h and fresh KGM was added in the presence of the test chemical. The cultures were allowed to incubate for an additional 18 h and the supernatants were collected for cytokine quantitation. Cell viability was determined by Trypan blue exclusion and confirmed by quantitating aliquots of the supernatants for the presence of lactate dehydrogenase (LDH). GM-CSF, IL-1α and IL-8 concentrations were determined by ELISA using commercially available systems and TNF-α concentrations measured by a cytolytic assay using L929 fibroblast cells with actinomycin D [5]. Immunoreactive TGF-α was determined from supernatants by Western blotting as previously described [6].

2.3. In vivo studies

Specific-pathogen-free BALB/c AnNCr female mice were obtained from Charles River Breeders (Raleigh, NC) at 7 weeks of age and used in the skin painting studies at 10–16 weeks of age. The test chemicals were dissolved in either distilled water, acetone:olive oil (4:1 v/v), or EtOH and applied slowly in 10-μl aliquots to the dorsal surface of both ears. The ear thickness was measured with an Oditest D 1000 micrometer (The Dyer Co., Lancaster, PA) at 3 and 24 h posttreatment. After the mice were asphxiated with CO_2, one ear from each mouse was removed, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Median sagittal sections (5 μm thick) were prepared and stained with hematoxylin and eosin for histologic examination.

In studies with sodium arsenite, we used the transgenic mouse line, TG.AC, which carries a v-Ha-*ras* oncogene fused to a zeta globin promoter [7]. The transgene confers on the skin the properties of a genetically initiated tissue. TPA and other promoters administered topically to the shaved dorsal skin or wounding of the skin readily induces squamous cell papillomas which can progress rapidly to squamous cell carcinomas or sarcomas in TG.AC mice [7,8]. By applying limited doses of TPA (2.5 μg; 2 times/week; 2 weeks) to the skin of TG.AC mice, a modest incidence of papillomas (mean of 3 per mouse) develop within 4 weeks (Spalding, unpublished observation). Wild-type FVB/N or TG.AC transgenic mice were given 0.02% arsenic (as sodium arsenite) in the drinking water and 4 weeks later TPA was applied to the skin on the dorsum. Papilloma development was monitored for up to 16 weeks following the initial exposure.

3. Results and discussion

Each of the dermatotoxicants were examined over a large concentration range to quantitate cytokine production in primary human keratinocyte cultures at cytotoxic and non-cytotoxic levels. If significant leakage of LDH occurred, it was thought that IL-1α might also be released and induce an autocrine stimulation of surviving cells. However, detectable increases in IL-1α were not observed in supernatants with most chemicals that induced significant cytotoxicity, the exceptions being high concentrations of croton oil and benzoalkonium (data not shown). The values shown for each chemical in Table 1 represent the change in cytokine secretion compared to control values (vehicle only) following 24 h of culture in the presence of the test chemical that produced the maximum change. Although there was no chemical class-specific cytokine profile, certain trends occurred which were noteworthy. First, the contact irritants provided the most vigorous proinflammatory cytokine response with the neutrophil chemokine, IL-8, readily detected. Neither the chemical sensitizers or ulcerative agents induced consistent increases in cytokine responses and, in fact, the latter class tended to inhibit secretion. This is in contrast to observations by us (data not shown) and others where proinflammatory cytokines are readily induced in the rodent skin following in vivo application and likely reflects the cellular interactions between T cells, keratinocytes and Langerhan cells that can occur in vivo [9,10]. In contrast to other chemicals examined, sodium arsenite induced significant secretion of the keratinocyte-derived growth factors, GM-CSF and TGF-α. A slight increase in TNF-α occurred, but there was no effect on IL-8 secretion by arsenic. In all cases chemical-induced increases in cytokine secretion coincided with changes in cytokine-specific mRNA as determined by reverse transcription-polymerase chain reaction RT-PCR (data not shown).

To determine whether the differences in cytokine profiles observed with the test chemicals were associated with specific histologic differences in vivo, the test agents were applied to the dorsal surface of mouse ears, and the skin was examined for swelling and histopathologic changes at 3 and 24 h (data not shown). All chemicals, except arsenic, induced edema, but the extent and time course for maximum ear swelling were chemical specific. Histologic evaluation indicated that benzoalkonium, ox-

Table 1
Effects of dermatotoxicants on cytokine secretion in cultures of human epidermal keratinocytes

Chemical (concentrations tested μM)	Cytokine increase[a]				
	IL-1α	TNF-α	IL-8	GM-CSF	TGF-α
Irritants					
Phenol (100–200)	3	2	11	1	ND
SLS (0.5–10)	12	1	2	ND	ND
Croton Oil (0.8–800)	5	5	112	16	12
Sensitizers					
DNFB (0.11–5)	1	1	1	1	ND
Oxazolone (0.08–40)	1	>1	>1	>1	ND
Ulcerative					
CrO_3 (0.05–5)	>1	>1	>1	>1	ND
Benzoalkonium (0.1–5)	1.4	>1	>1	>1	ND
Carcinogenic					
Arsenic (0.2–8)	2	3	1	8	3

[a] These values represent maximum-fold increases or decreases from control supernatants (no test chemical added). Cytokine responses less than 1.0× indicate a suppression of baseline cytokine production. Cytokine responses of 1.0× indicate that there was no change in the baseline production. Cytokine responses greater than 1.0× indicate an induction of a given cytokine. ND, not determined.

azolone and croton oil produced relatively severe and progressive changes for at least 24 h. Benzoalkonium caused patchy epithelial necrosis followed by edema, leukostasis, perivascular cuffing, and necrotizing angitis with a mild extravasation of erythrocytes. Oxazolone affected only selected areas, characterized by a mild epidermal necrosis at the orifice of hair follicles, which was followed by an accumulation of neutrophils in the epidermal layer where the necrosis first occurred. Croton oil did not induce necrosis, but caused edema and mild leukostasis, which was followed by a diffuse, intensive subepidermal and dermal neutrophil infiltration. SLS, phenol and CrO_3 induced mild to moderate transient changes that peaked within 3 h. Dinitrofluorobenzene (DNFB) and SLS did not induce necrosis, but DNFB induced severe edema and lymphangiectasia in which there was little neutrophil infiltration. Phenol application induced an extensive, although patchy, epidermal necrosis, which extended in some areas to the subepidermis and resembled local ischemia. Neutrophil infiltration was mild to moderate in these regions as evidenced by leukostasis and perivascular cuffing detected at 3 h, whereas the edema was moderate. Of particular note, it was observed that the chemical irritants, all of which induced IL-8, revealed a neutrophil gradient following in vivo application while those chemicals that did not produce the neutrophil chemokine presented a diffuse neutrophil pattern.

Taken together, these studies suggest that the generation and secretion of IL-8 may provide a useful biomarker for determining skin irritants. However, the cytokine profiles induced by sensitizers and ulcerative agents in keratinocyte cultures was not predictive of their in vivo cutaneous toxicity and reflects the fact that multiple mechanisms and cell types are involved in the induction of these dermatotoxic responses. Determining the source, kinetics of production, and the regulation of inflammatory mediators in the skin will be of value in predicting various toxicities arising from exposure to environmental agents and may be helpful in the development of strategies for the treatment of environmentally based skin diseases.

Both TGF-α and GM-CSF, as keratinocyte growth factors, have been associated with the development of skin papillomas (review in [6]). As these growth factors, particularly TGF-α, can

serve as tumor enhancers [11,12] and arsenic induces their secretion, the influence of arsenic on mouse skin tumor development was studied in transgenic TG.AC mice which carry a v-Ha-*ras* oncogene. As shown in Fig. 1, within 6 weeks following TPA, the incidence of papillomas in arsenic-exposed, TPA-treated TG.AC mice was approximately 4-fold higher than in promoted mice that had not received arsenic. The incidence decreased to 2-fold 10 weeks following TPA treatment due to the death of animals with heavy papilloma burdens (not shown). Papillomas were not present in non-promoted TG.AC mice or wild-type FVB/N mice that had received arsenic when observed up to 3 months. These results suggest that arsenic can serve as a tumor enhancer but not a complete carcinogen or promoter. The histopathology assessment of the papillomas was similar to that reported previously in TG.AC mice promoted with TPA [7] and were predominantly squamous cell papillomas (data not shown). Consistent with these observations, 2 cell types in arsenic-induced skin tumors have been identified in humans: basal cell carcinomas and squamous cell carcinomas arising in keratotic areas. The basal cell carcinomas are usually only locally invasive while squamous cell carcinomas may have distinct metastases.

Considering the accumulating evidence that keratinocyte growth factors enhance skin tumor formation, the present data suggest that arsenic exerts its carcinogenicity through a similar mechanism involving chronic, low-level stimulation of keratinocyte-derived growth factors coupled with possible genetic effects. The high affinity of arsenic for sulfhydryl groups leads to its accumulation and tenacious retention in keratin-rich tissues such as hair and skin, and tissue levels of arsenic have been used as a quantitative indicator of exposure in humans [13]. Analysis of hair samples collected from mice in the present studies showed that arsenic reached an average content of 329 ng/g with 4 weeks following exposure compared to 0.6 ng/g for controls (data not shown). In addition to studies of arsenic carcinogenicity, the present finding suggest that

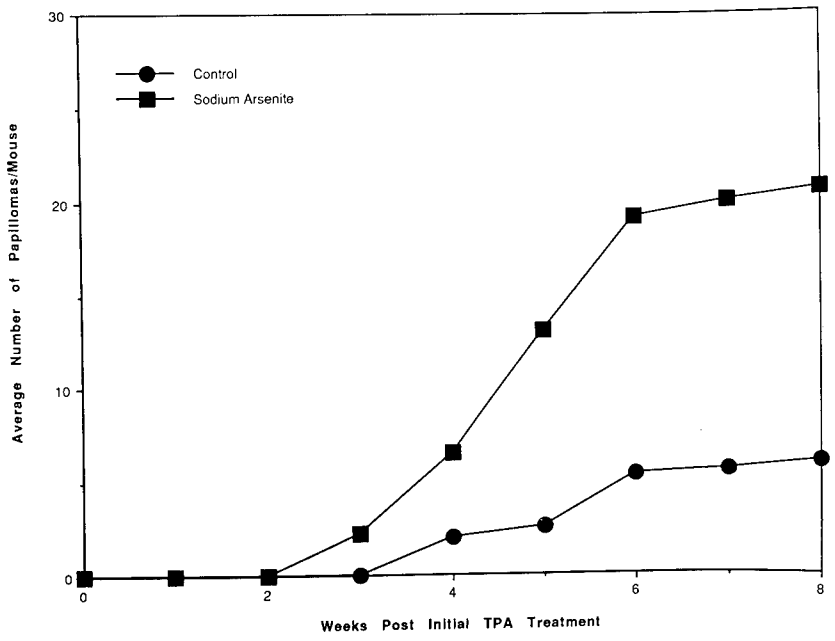


Fig. 1. Papilloma incidences in TG.AC transgenic mice given 0.02% sodium arsenite in the drinking water starting at 12 weeks of age. Mice were pretreated with arsenite or water for 4 weeks and sub-groups were treated 4 times with 2.5 μg TPA in acetone (administered twice per week for 2 consecutive weeks). Papillomas were not observed in non-TPA-promoted, arsenic-treated transgenic mice or wild-type FVB/N mice (not shown). $n = 20$ per group.

cultured human epidermal keratinocytes and the TG.AC transgenic mice may be useful models for assessing the tumor-enhancing properties of chemical carcinogens.

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Role of proinflammatory cytokines in a toxin response: application of cytokine knockout mice in toxicological research

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Abstract

The toxic effect of xenobiotics may be direct or mediated through the release of secondary toxic mediators such as proinflammatory cytokines. Knockout mice (KO or −/−) with discrete gene deletions obtained by homologous recombination may prove the function of a given cytokine in general and in toxico-pathogenesis. The technology is briefly described, and a short review of the phenotype of mice with deletions of several proinflammatory cytokines is given. The emphasis of this presentation is on the role of tumor necrosis factor, interleukins and interferon-γ in endotoxic shock and acute phase response. The future application of these mice for mechanistic studies in toxicological research is discussed.

Keywords: Knockout-mice; Endotoxin; TNF; Interferon; IL-1; ICE; IL-12

1. Introduction

Cytokines are small-molecular-weight proteins acting often locally in the tissues in an autocrine or paracrine fashion, but also a systemic activity is observed. They comprise regulatory factors of the immune system, hemopoiesis, tissue repair and inflammation. A few cytokines are recognized as mediators of toxicity, which include the proinflammatory cytokines tumor necrosis factor (TNF), interferon-γ (IFNγ), leukemia inhibitory factor (LIF) and interleukin-1 (IL-1), IL-6 and IL-12.

Two subsets of T helper (Th) lymphocytes can be distinguished based on the cytokines they produce and on their function [1]. Th1 cells produce IL-2, lymphotoxin (LT-α), IFNγ and IL-12, and promote a cellular or delayed-type hypersensitivity reaction, while Th2 cells produce IL-4, IL-5 and IL-10, and induce a humoral immune response with antibody production. Furthermore, Th1-derived cytokines such as IFNγ and IL-12 inhibit a Th2 response, while Th2-derived IL-4 and IL-10 inhibit a Th1 response and the production of Th1 cytokines.

The physiological role of a cytokine can be assessed by administering neutralizing antibodies, an approach which has been extensively exploited in the area of TNF and IFNγ [2]. More

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definitive answers may be obtained by inactivating a gene of interest, by homologous recombination in embryonic stem cells.

2. Homologous recombination, basic technique

This recent technology allows the disruption of given genes in embryonic stem cells by homologous recombination, permitting the development of mice with specific genetic modification [3]. The targeted embryonic stem cells are injected into blastocysts, which are implanted in a pseudopregnant foster mother allowing the development of chimeric mice. Breeding to homozygosity results in mice with gene deletions (−/−, knockout mice). In most instances, the deletion of single cytokine genes is – with a few exceptions – compatible with normal life, suggesting that the system has a high degree of redundancy. It should be mentioned here that nature preceded the ingenous molecular biologists providing us with murine strains containing distinct cytokine deficiencies. The classical examples are the osteopetrotic mouse (op/op), which lacks M-CSF, and the W/W and steel mouse which lack either the receptor (c-kit) of the stem cell factor (SCF) or its ligand, SCF. Other interesting examples are mutation in Fas and Fas ligand in the lpr and gld mice respectively, cytokines which belong to the TNF family.

3. Overview of the phenotype of cytokine knockout mice

In the last few years, several cytokines have been deleted by homologous recombination (Ryffel, in preparation). The phenotype of mice with deletions of several proinflammatory cytokines such as IL-1 and IFN is given with emphasis on our own work on TNF inactivation [4].

4. TNF family

TNF, LT-α and -β are members of a larger family of related proteins, which have prominent inflammatory, cytotoxic, vascular and immunoregulatory effects. TNF, LT-α and -β are clustered within the MHC class II locus on murine chromosome 6. The membrane TNF precursor is cleaved by a yet unidentified metalloproteinase to the soluble TNF which forms homotrimers [5]. While TNF and LT-α are secreted by cells, LT-β occurs only as a membrane-bound form. TNF and LT-α form homotrimers and bind to 2 distinct receptors, TNF-R I and II with mol. wt. of 55 and 75 kDa, respectively. LT-α and the membrane-bound LT-β form a heterotrimer [6] which signals through a novel receptor, the LT-$\alpha\beta$-R [7]. The deletion of distinct genes in this system allowed a better understanding of their functions, which are described:

4.1. TNF receptor knockout

TNF-R I-deficient mice are resistant to the lethal effect of low doses of endotoxin (lipopolysaccharide; LPS) after sensitization with D-galactosamine and have an increased susceptibility to the facultative intracellular bacterium *Listeria monocytogenes* [8,9]. Therefore, the TNF-R I plays a decisive role in host defense against microorganisms and their pathogenic factors. TNF-R II-deficient mice are normal except for a increased sensitivity to TNF [10].

4.2. LT-α- and TNF-LT-α double knockout

LT-α-deficient mice demonstrated an unexpected phenotype [11]: mutant mice had no detectable lymph nodes and Peyer's patches, dysmorphic spleen architecture with a normal thymic architecture. These data suggest an essential role for LT-α in the development of peripheral lymphoid organs.

The simultaneous deletion of TNF and LT-α inactivates all presently known pathways and represents a complete knockout interrupting signalling through the TNF-R I and II as well as the LT-$\alpha\beta$-R [4]. The TNF/LT-α knockout combines therefore the phenotypes of TNF-R I (endotoxin resistance and susceptibility to *Listeria* infection) and LT-α deficiency (absence of peripheral lymphoid organs). The double knockout however has additional features, e.g. absence of an IgA mucosal immune system, low IgG and IgA serum levels, defective IgG class switch in response to a T-cell-dependent antigen, distinct B lymphocytosis with perivascular lymphoid infil-

tration in several organs, upregulation of major histocompatiblity class I antigens and reduced generation of cytotoxic T cells [4]. These data suggest that the combined lack of TNF and LT-α has profound developmental and functional effects on the immune system.

5. IL-1

The IL-1 family have proinflammatory properties and comprise the agonistic IL-1 α and β and the antagonistic IL-1 receptor antagonist, which signal through the type I IL-1 receptor. Inactivation of the IL-1 genes has been recently achieved: mice with disrupted IL-1 ligands develop normally, and they appear to be sensitive to endotoxic shock (unpublished). Related to IL-1 is the IL-1β converting enzyme (ICE), which is a cysteine protease and processes the inactive 31-kDa IL-1β precursor to the active, proinflammatory 17-kD mature IL-1β. Upon endotoxin stimulation mice with disrupted ICE gene do not produce IL-1β or IL-1α and have a reduced synthesis of TNF and IL-6. The mutant mice are resistant to endotoxic shock [12,13].

6. IL-6

IL-6 is a multifunctional cytokine with regulatory effects on B lymphocytes, hematopoiesis, inflammation and acute phase response.

Overexpression of IL-6 in mice resulted in plasmacytosis and mesangioproliferative glomerulonephritis [14]. Obviously, these findings provoked extensive safety studies. High doses of rhIL-6 given to rodents or non-human primates induced thrombocytosis and increased synthesis of acute phase proteins without any signs of systemic toxicity [15,16].

IL-6-deficient mice develop normally. However, IL-6 $-/-$ mice fail to control efficiently vaccinia virus and *Listeria monocytogenes* infection. The inflammatory acute phase response after tissue damage or infection is severely compromised, whereas it is only moderately reduced after injection with endotoxin [17,18].

7. IFN

IFN comprise a large family of proteins: IFNα and β (type I IFN), with antiviral and antiproliferative properties, and IFNγ (type II or immune IFN) with immunoregulatory roles. IFNγ is produced by T helper (Th) cells and natural killer (NK) cells and activates Th1 lymphocytes, macrophages, NK cells and B cells. The role of IFNγ in macrophage activation and hence in inflammation is now fully recognized. The disruption of the gene for IFNγ or its receptor allowed an unequivocal assignment of the in vivo IFNγ functions.

7.1. IFNγ receptor knockout

Mice with disruption of the IFNγ-R gene develop normally and have normal IFNγ synthesis, but are unresponsive to it. IFNγ-R-deficent mice have clearly a defective natural resistance: they demonstrate increased susceptibility to infections by *Listeria monocytogenes* and vaccinia virus despite normal cytotoxic and Th cell responses [19]. Further, IFNγ seems to be necessary for an antigen-specific immunoglobulin G2a response and for an inflammatory response. Therefore, disruption of the IFNγ signalling causes a profound impairment of natural resistance and macrophage functions.

8. IL-12

IL-12 is a macrophage-derived heterodimeric cytokine with potent immunostimulatory effects. IL-12 activates T and NK cells, which synthesize several cytokines including IFNγ and activate macrophages [20]. Inactivation of either component of the IL-12 heterodimer (p35 or p40) allows normal development, but a diminished host response is expected (unpublished).

The activity and toxicity of IL-12 may be in part related to its ability to induce the synthesis of IFNγ. In mice, a striking feature is anemia and leukopenia after IL-12 administration. Therefore, we investigated the effect of IL-12 on hemopoiesis in mice with inactivated IFNγ-R gene [19]. We show that the bone marrow de-

pression induced by IL-12 in wild-type mice depends on IL-12-induced IFNγ [21].

8.1. IL-12-induced hepatitis

IL-12 recruits activated T cells, macrophages and NK cells into the liver resulting in hepatitis, which depends on IL-12-induced endogenous IFNγ. IL-12-induced hepatitis was largely abrogated in IFNγ-R deficient mice, which are unresponsive to IFNγ [22].

9. Pathophysiology of endotoxic shock

Based on the results of many recent investigations it appears that several factors play an important role in the pathogenesis of endotoxic shock among which figure TNF, IL-1, IFNγ, adhesion molecules, nitric oxide.

A central role of TNF in endotoxic shock was demonstrated by several ways. Administration of neutralizing TNF antibodies or soluble receptors [23], inhibition of TNF synthesis [24] or release of TNF or by inhibiting the membrane metalloproteinase necessary for cleavage of membrane bound TNF [5], all measures prevent the systemic bioavailability of TNF and therefore inhibit lethal endotoxic shock in mice. Thus, resistance to endotoxic shock was correctly predicted in TNF-R I and TNF/LT mice [4,8,9].

The role of IFNγ in endotoxic shock was assessed in IFNγ receptor-deficient (IFN-γR) mutant mice [19]. We reported increased resistance to LPS resistance in mutant mice, tolerating 100 times higher doses of LPS than the minimum lethal dose for wild-type mice after sensitization with D-galactosamine (D-GalN). The rise in hepatic enzymes and hepatocellular degeneration were attenuated in IFNγR$-/-$ mice. Serum TNF levels were about 10-fold reduced in mutant mice given LPS or LPS/D-GalN. Therefore, IFNγ is a critical mediator of endotoxic shock. Resistance to endotoxin in IFNγR$-/-$ mice, which have a disrupted IFNγ-signalling system, is likely due to depressed TNF synthesis, reduced LPS receptor expression and absence of a direct toxic effect of IFNγ [25].

ICE plays an important role in the generation of bioactive IL-1. Mice with disrupted ICE gene do not produce IL-1β or IL-1α and produce only little IL-6 and TNF after stimulation with LPS. The mutant mice are therefore resistant to endotoxic shock [12,13].

Deletion of adhesion molecules such as ICAM-1, selectins [26–29] as well as deletion of the chemotactic receptor [30] affects the extravasation of leukocytes and as a result the mice are more resistant to endotoxin (LPS), despite normal production of TNF and IL-1.

Nitric oxide synthase (NOS) is induced in endotoxemia, and the vasodilating NO is released into the circulation. Since NOS-deficient mice display increased resistance to LPS-induced shock, NO likely is involved in the pathogenesis [31,32].

LIF is also induced in endotoxic shock and likely plays a role. Since LIF-deficient mice are more sensitive to endotoxic shock, these data suggest that LIF has in contrast to TNF a protective effect in endotoxic shock (Weber et al., submitted).

10. Acute phase reaction

Upon tissue injury, trauma or infection, a complex series of reactions occur in the host in an effort to prevent ongoing damage, isolate and destroy infective organisms and activate the repair process. This well-orchestrated immediate and early reaction to inflammation is known as the acute phase response (APR). An APR comprises fever, release of several vasoactive and chemotactic factors, arachidonic acid metabolites and the rapid and massive synthesis of hepatic acute phase proteins (APP), e.g. serum amyloid protein, C-reactive protein, α2-macroglobulin, etc. The synthesis of APP is regulated by type I (IL-1, TNF, LT) and type II (IL-6, IL-11, LIF) cytokines, glucocorticosteroids and insulin [33]. As indicated before mice with deletion of the inducing genes have reduced synthesis of APP. The mutant mice will allow a better understanding of the biological and especially of possible protective role of APP in toxicity.

11. Conclusion and perspective

This short review on knockout mice demonstrates the function of some proinflammatory cytokines. In order to use such animal models it is important that the disruption of a single gene does not perturb the complex biological systems. In most cases deletion of a single cytokine gene allowed normal development of healthy mice. Therefore, such animals are excellent tools to investigate the role of a given gene, which may be induced by a toxin for toxico-pathogenesis. The role of TNF and IFNγ in several pathological and toxicological conditions was highlighted, especially in endotoxin and IL-12 toxicity. The use of knockout mice will be instrumental for mechanistic studies in the future.

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Toxicology Letters

TNFα and increased chemokine expression in rat lung after particle exposure

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Abstract

Macrophage inflammatory protein 2 (MIP-2) and CINC (**C**ytokine-**I**nduced-**N**eutrophil-**C**hemoattractant) are members of the chemokine family of inflammatory and immunoregulatory cytokines. MIP-2 and CINC exhibit potent neutrophil chemotactic activity and are thought to be key mediators of inflammatory cell recruitment in response to tissue injury and infection. In the present studies, we examined the potential involvement of MIP-2 and CINC in particle-elicited inflammation in the rat lung and the role of TNFα in particle-induced chemokine expression. Acute intratracheal instillation exposure of F344 rats to α quartz or titanium dioxide was shown to markedly increase steady-state levels of MIP-2 and CINC mRNA in lung tissue; a response which was associated with a significant increase in neutrophils in the bronchoalveolar lavage fluid. Additional studies demonstrated that acute inhalation of crocidolite fibers by rats also induced increased MIP-2 and CINC expression. Since previous studies had demonstrated that TNFα stimulates MIP-2 and CINC expression in vitro and that particle exposure induces TNFα production in rat lung we examined the role of TNFα in α quartz-induced MIP-2 gene expression. We demonstrated that passive immunization of mice against TNFα markedly attenuated the increased lung MIP-2 mRNA seen in response to α quartz inhalation. Collectively, these findings suggest that the chemokines MIP-2 and CINC play a role in neutrophil recruitment to the rat lung after particle exposure and indicate that particle-induced expression of these chemokines is mediated, at least in part, by production of TNFα.

Keywords: Chemokines; Macrophage inflammatory proteins; CINC; Silica; Crocidolite; Asbestos; Neutrophils

1. Introduction

MIP-2 was originally identified as an approximately 6-kDa protein secreted by endotoxin-stimulated mouse macrophages and shown to exhibit neutrophil chemotactic activity in vitro and elicit a neutrophilic infiltrate when injected into the hind foot pads of mice [1]. CINC was first characterized as an IL-1-inducible neutrophil chemotactic cytokine released by a rat kidney epithelial cell line [2]. MIP-2 and CINC show a high degree of structural and functional homology and are recognized as members of a supergene family of inflammatory and immuno-

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regulatory cytokines known as chemokines [3,4]. MIP-2 and CINC can be derived from a variety of cell types including macrophages, neutrophils, epithelial cells and fibroblasts in response to stimuli, such as: endotoxin; the proinflammatory cytokines, TNFα and IL-1; as well as the protein kinase C activator phorbol myristate acetate (PMA) [4,5]. An increasing number of studies suggest that members of the chemokine cytokine family, including MIP-2 and CINC, contribute to respiratory tract defense mechanisms [6]. In this respect, it has been proposed that a key pathway of inflammatory cell recruitment to the lung involves a cytokine network in which TNFα produced by macrophages or other lung cells stimulates chemokine production; the chemokines then act as the proximate mediators of cell recruitment [6–9].

Inhalation of noxious particles can result in pulmonary inflammation characterized by increases in macrophages and neutrophils [10]. The elicited inflammatory cells play an important role in host defense but can also contribute to lung injury through the release of tissue damaging proteases and oxidants [11–13]. At present, the mechanisms underlying the recruitment of inflammatory cells after particle exposure remain incompletely understood; however, recent studies suggest chemokines play an important role [5,6]. To better understand the processes underlying particle-elicited lung inflammation, we characterized the expression of MIP-2 and CINC mRNA in the rat lung after exposure to α quartz, titanium dioxide and crocidolite, particulate materials which we have previously shown to increase production of TNFα by alveolar macrophages [5,13]. In addition, using a passive immunization model we investigated the role of TNFα in particle-induced increases in rat lung chemokine expression.

2. Materials and methods

2.1. α Quartz and titanium dioxide intratracheal instillation studies

As described elsewhere in detail [5], groups of male F344 rats were intratracheally instilled with saline or saline suspensions of α quartz (minusil; mean diameter = 2.1 ± 1.1 μm; surface area = 4.5 m^2/g) or titanium dioxide (TiO_2-F) (from Fisher Scientific; diameter, ~0.25 μm; surface area = 8.8 m^2/g) at a dose level of 2 mg/rat. The mineral dusts were heated at 200°C for 2 h prior to use for sterilization and inactivation of any endotoxin present. At 2, 6 and 24 h after exposure animals were killed by intraperitoneal (i.p.) injection of Na pentobarbital (50 mg/kg) and exsanguination via the abdominal aorta. The lungs of two animals/treatment/time were removed enbloc, trimmed of extraneous tissue and quick frozen in liquid nitrogen for later isolation of RNA. RNA was extracted as described by Chomczynski and Sacchi [14] and analyzed using RT-PCR for MIP-2 and CINC mRNA expression.

2.2. Inhalation of crocidolite asbestos

Male Fischer F344 rats were exposed via inhalation to crocidolite asbestos fibers as described in detail by Quinlan et al. [19]. Briefly, animals were exposed to National Institute of Environmental Health Sciences (NIEHS) reference samples of crocidolite at concentrations of 0.1 mg/m^3 (approximately 60 fibers >5 μm long/cc air) and 8 mg/m^3 (approximately 2800 fibers >5 μm lung/cc air). Animals were given either a 3-h or a 6-h exposure to crocidolite followed by 18 h in room air. They were then killed by i.p. injection of Na pentobarbital, the lungs lavaged and the number and type of lavage cells determined. In addition, lungs were isolated and RNA extracted as described by Chomczynski and Sacchi [14] and analyzed using RT-PCR for MIP-2 and CINC mRNA expression.

2.3. TNFα passive immunization studies

Mice were used in the passive immunization studies to minimize the quantity of anti-sera needed. Briefly, groups of two C57Bl mice were given i.p. injections of 0.5 ml saline, normal rabbit serum or rabbit anti-mouse TNFα antisera. Eighteen hours after i.p. injection the animals were exposed to air or an aerosol of 100

mg/m^3 α quartz for 6 h. The generation and characterization of α quartz was as described previously [15]. Six hours after air sham or α quartz exposure, animals were killed by i.p. injection of Na pentobarbital (50 mg/kg), the lungs removed and RNA extracted for RT-PCR analysis of MIP-2 mRNA expression.

2.4. Chemokine expression

MIP-2 and CINC mRNA transcript levels were assessed by PCR amplification of the corresponding cDNAs as described in detail elsewhere [5]. β-Actin or GAPDH mRNA was evaluated concurrent with chemokine mRNA as an internal control. Total lung RNA from a normal rat and from a rat instilled with 10 μg LPS and sacrificed 6 h after exposure was analyzed concurrent with unknown RNA samples as positive and negative controls for chemokine mRNA expression. The primers were designed from the published sequences for MIP-2 [24], CINC [16], β-actin [17] and GAPDH [18] and were the following:
MIP-2: 5′-GGCACATCAGGTACGATCCAG-3′; 5′-ACCCTGCCAAGGGTTGACTTC-3′;
CINC: 5′-TTCTCTGTGCAGCGCTGCTG-3′; 5′-CAGGGTCAAGGCAAGCCTCG-3′;
β-actin: 5′-CTCATAGATGGGCACAGTGTG-3′; 5′-AACCGGTCCGCCATGTGCAAG-3′;
GAPDH: 5′-CAGGATGCATTGCTGACAAT-C-3′; 5′-GGTCGGTGTGAACGGATTTG-3′.

3. Results

3.1. Effect of dust exposure on lung inflammation and chemokine expression

The changes in lung MIP-2 mRNA expression and BAL cell number after intratracheal instillation with α quartz and titanium dioxide are described in detail elsewhere [5]. Minimal or no mRNA for MIP-2 and CINC were detected in the lungs of saline instilled control rats (Fig. 1a–c). Acute exposure to α quartz and titanium dioxide increased steady-state levels of mRNA for MIP-2 and CINC. Increased MIP-2 and CINC mRNA was detected by 2 h after dust exposure and persisted until 24 h, although the levels of mRNA appeared to decrease by 24 h after exposure particularly for the titanium dioxide-exposed rats. Intratracheal instillation of 2 mg α quartz and titanium dioxide resulted in a significant increase in the number of neutrophils in BAL fluid ($P < 0.05$; ANOVA followed by Dunnett's test) 6 and 24 h after dust exposure (Fig. 1d); no significant increase in macrophages were detected.

As described previously by Quinlan et al. [19], exposure of rats for 3 or 6 h to 8 mg/m^3 crocidolite fibers did not produce an increase in total cell numbers in BAL but did result in a significant increase in the proportion of neutrophils in the BAL cell population (Fig. 2). Exposure to 0.1 mg/m^3 did not elicit any detectable change in the numbers or types of cells in BAL fluid. mRNA for MIP-2 or CINC was not detected in air control rats (Fig. 3) but was present in the lungs of rats exposed for 3 or 6 h to 10 mg/m^3. Only CINC mRNA was detected in rat lungs after 3- or 6-h exposures to 0.1 mg/m^3 crocidolite.

3.2. Effect of passive immunization against TNFα on MIP-2 gene expression

To examine the relationship between particle-induced TNFα release and BAL fluid neutrophils we conducted a study in which mice were passively immunized with anti-TNFα antibody and given an acute inhalation exposure to α quartz. No mRNA for MIP-2 was detected in lungs of mice pretreated with saline, rabbit anti-murine TNFα antisera or nonimmune rabbit sera and given an air-sham exposure (data not shown). In animals pretreated with saline or nonimmune rabbit sera and exposed to α quartz, MIP-2 mRNA expression could be clearly detected (Fig. 4). In contrast, in animals exposed to anti-TNFα antisera there was an attenuation of the α quartz-induced increase in MIP-2 mRNA (Fig. 4).

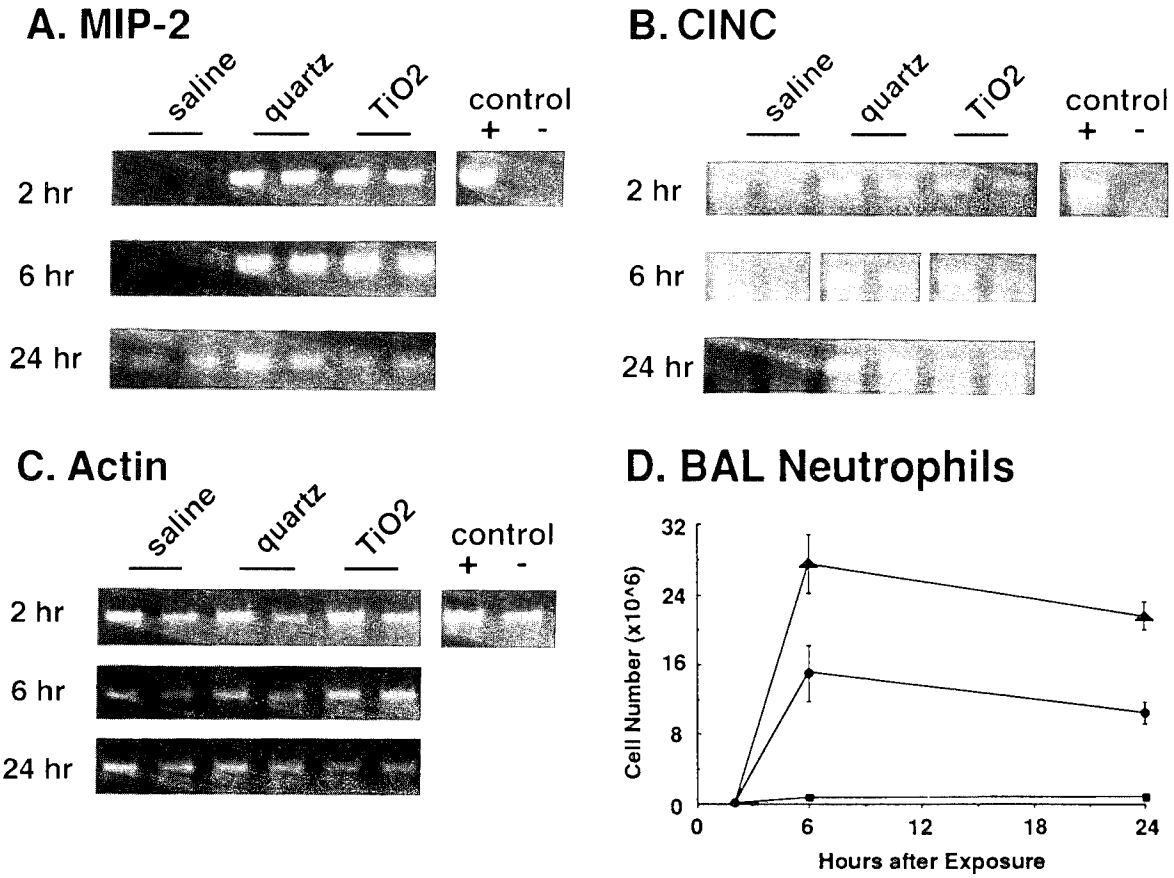


Fig. 1. MIP-2 and CINC mRNA expression in rats lungs after intratracheal instillation of α quartz and titanium dioxide (TiO_2). Shown are ethidium bromide-stained gels for (A) MIP-2, (B) CINC and (C) β-actin PCR products ($N = 2$ rats) amplified from 1 μg RNA from the lungs of rats 2, 6 and 24 h after intratracheal instillation of 10 mg/kg body weight α quartz or TiO_2 particles. (+) and (−) controls represent lung RNA from LPS instilled and normal rats, respectively. (D) Numbers of neutrophils obtained by bronchoalveolar lavage of rats ($N = 4$) 2, 6 and 24 h after intratracheal instillation of saline or 10 mg/kg body weight α quartz and TiO_2; squares, saline control; triangles, α quartz; circles, TiO_2 (modified from [5].

4. Discussion

The recruitment of inflammatory cells to the respiratory tract is an important component of host defense. Cells such as macrophages and neutrophils can facilitate the pulmonary clearance and inactivation of inhaled particles and pathogens [20]. Inflammatory cells, however, can also contribute to the pathogenesis of lung disease through the excessive release of reactive oxygen species and proteolytic enzymes which can damage lung tissue. In this respect, understanding the mechanisms by which particles and other inhaled materials stimulate the recruitment of inflammatory cells should provide information on the regulation of an important host defense mechanism as well as insight into the processes which can contribute to the pathogenesis of particle-induced lung disease. In the present studies we provide evidence that two members of the chemokine cytokine family, MIP-2 and CINC, are likely mediators of particle-elicited inflammatory cell recruitment to the rat lung. In addition, we demonstrate that particle-induced expression of MIP-2 is dependent, at least in part, on early release of the pro-inflammatory cytokine TNFα. These findings provide the first in vivo data supporting a mechanism of particle-

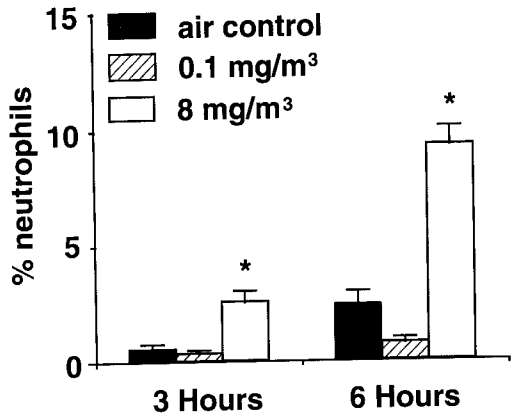


Fig. 2. Effect of acute crocidolite fiber inhalation on neutrophilic inflammation in the rat lung. Shown are the proportions of neutrophils in bronchoalveolar lavage fluid from rats exposed for 3 or 6 h to air or to 0.1 and 8 mg/m^3 crocidolite asbestos fibers. Results represent the X ± S.E. of groups of three rats/exposure; *statistically significant differences from the air control group; $P < 0.05$ (from Ref. [19]).

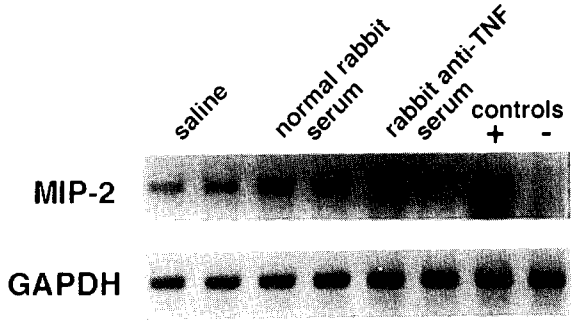


Fig. 4. Effect of passive immunization of mice with anti-TNFα antisera on α quartz-induced MIP-2 expression. Briefly, C57Bl mice were pretreated with 0.5 ml of saline, normal rabbit sera or rabbit anti-mouse TNFα sera and 18 h later exposed to an aerosol of 100 mg/m^3 α quartz for 6 h. Six hours after dust exposure, animals were sacrificed and expression of MIP-2 mRNA in the lungs determined. Shown are ethidium bromide-stained gels presenting the MIP-2 and GAPDH PCR products ($N = 2$ rats) amplified from 1 μg of mouse lung RNA.

elicited lung inflammation which involves the early release of TNFα and subsequent activation of chemokine production.

Previous studies have indicated that the chemokines MIP-2 and CINC may be important mediators of inflammation after dermal wounding, in pulmonary sepsis as well as in alcohol-induced liver injury [4]. In the present study we demonstrate that acute exposure of rats to α quartz and titanium dioxide particles increases steady-state levels of MIP-2 and CINC mRNA in lung tissue; a response associated with neutrophilic inflammation in the lung. Given MIP-2 and CINC are known to be potent neutrophil chemotactic factors, the marked increase in their expression after α quartz and titanium dioxide

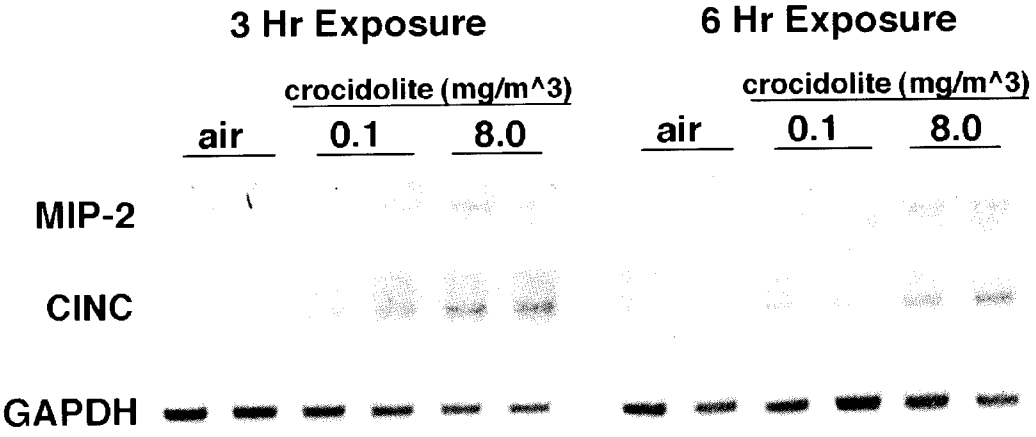


Fig. 3. Effect of acute crocidolite fiber inhalation on rat lung expression of MIP-2 and CINC mRNA. Rats were exposed for 3 or 6 h to air or to 0.1 and 8 mg/m^3 crocidolite asbestos fibers and steady-state levels of MIP-2 and CINC mRNA in lung tissue determined. Shown are ethidium bromide-stained gels presenting the MIP-2, CINC and GAPDH PCR products ($N = 2$ rats) amplified from 1 μg of rat lung RNA.

suggests they contribute to the inflammatory response elicited by these dusts. In addition to α quartz and titanium dioxide, we also examined chemokine expression in rat lung after acute inhalation of crocidolite fibers, since this crocidolite has been shown to result in significant neutrophilic inflammation in the rat [19,21]. Similar to the other particles, exposure to crocidolite increased steady-state levels of MIP-2 and CINC mRNA in the lung. Both MIP-2 and CINC expression were observed at the 10 mg/m^3 exposure and this response was associated with neutrophilic inflammation. However, at the low fiber concentration, 0.1 mg/m^3, only slight expression of CINC mRNA was detected and this was not associated with increased neutrophils suggesting that the level of CINC was insufficient to elicit an inflammatory response.

At present the mechanisms by which in vivo exposure to α quartz, titanium dioxide or crocidolite stimulate chemokine production in the rat lung is unknown. However, there is evidence that the pro-inflammation cytokine TNFα plays an important role. As mentioned above, TNFα can stimulate release of both MIP-2 and CINC from a variety of cell types. In this respect, it has been suggested that an important mechanism of cell recruitment to the lung involves the initial production of TNFα which then acts via autocrine or paracrine pathways to activate lung cells to release chemokines which provide the chemotactic stimulus for cell recruitment [4,6–9]. There are several lines of evidence supporting a role for TNFα as a key factor in particle elicited pulmonary inflammation. For example, studies with α quartz, titanium dioxide and crocidolite exposed rats have demonstrated a significant positive correlation between in vivo activation of macrophage TNFα production and the degree of neutrophilic inflammation in the lung [5,13]. Similarly, a positive correlation has been reported between BAL fluid TNFα levels and neutrophilic inflammation in the lungs of humans exposed to asbestos or patients with idiopathic pulmonary fibrosis [22]. In addition, passive immunization studies have demonstrated that pretreatment of rats with antibody to TNFα markedly attenuates the rat lung inflammatory response to titanium dioxide or α quartz [6,23]. The demonstration in the present studies that passive immunization of mice against TNFα attenuates α quartz-induced MIP-2 gene expression provides the first in vivo evidence that TNFα, in fact, facilitates neutrophil recruitment by inducing expression of chemokines. Overall, these new observations, combined with existing information on TNFα production after particle exposure provides compelling evidence that a TNFα-mediated cytokine network is one of the pathways underlying particle-elicited inflammatory cell recruitment.

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Cytokine regulation of chemical sensitization

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Abstract

The skin is an immunologically active tissue. Epidermal cells, both keratinocytes and Langerhans cells (LC), produce constitutively or can be stimulated to produce a variety of cytokines, many of which play important roles in the induction and regulation of allergic responses to sensitizing chemicals. Tumor necrosis factor α (TNF-α) provides the signal for LC migration from the skin and granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin 1 (IL-1) and other cytokines effect the functional maturation of LC and their acquisition of immunostimulatory potential. The initial stimulus for induced or increased epidermal cytokine production derives from chemical exposure, or some other form of skin trauma. However, some epidermal cytokines are regulated in paracrine or autocrine fashion by other cytokines produced locally. The availability of epidermal cytokines has a major impact on the induction of sensitization and on the characteristics of immune responses to chemical allergens.

Keywords: Sensitization; Epidermal cytokines; Langerhans cells; T helper cells

1. Introduction

The skin is by definition the route of exposure for contact sensitization. There is evidence also that certain chemicals when encountered on the skin may provoke the quality of immune response necessary for effective sensitization of the respiratory tract. The skin is an immunologically active tissue and in the context of allergic sensitization to chemicals its importance is in transducing the antigenic signal to regional lymph nodes. Stimulation of cutaneous immune responses is dependent upon the activity of dendritic cells (DC) and the cytokines that serve to regulate their behaviour. The cellular and molecular interactions necessary for the induction of sensitization and that determine the characteristics of skin immune responses are the subject of this article.

2. Langerhans cells

Langerhans cells (LC) are bone marrow-derived DC that form a semi-contiguous network in the epidermis. Here they serve as a trap for antigens encountered on the skin. LC resident in the epidermis are able effectively to internalize and process antigen in the manner required for subsequent stimulation of immune responses [1,2]. Immune activation takes place in regional lymph nodes and an important function of LC is the transport of antigen from the skin to lymph nodes draining the site of exposure. During this process of migration from the epidermis, via the afferent lymphatics, LC are subject to phenotypic changes such that by the time the local lymph

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nodes are reached they have acquired the characteristics of mature DC and are able to present antigen to responsive T lymphocytes [3]. Thus, in response to skin sensitization there is a rapid accumulation of DC, including allergen-bearing DC, in draining nodes. These cells are functionally active and, unlike LC found within the epidermis, will stimulate vigorous immune responses [3,4]. It is apparent, therefore, that LC exist in 2 discrete forms. In the skin LC recognize and process antigen. Subsequently, as they migrate from the skin the capacity for antigen processing is lost and exchanged for antigen-presenting potential [1,3]. The acquisition of immunostimulatory activity is achieved in part through the induced expression of membrane proteins and glycoproteins necessary for presentation of antigen to T lymphocytes. It has been shown for instance that, compared with epidermal LC, the antigen-bearing cells that accumulate in draining lymph nodes following skin sensitization display a substantial (5-fold) increase in the expression of MHC class II (Ia) antigens; the membrane determinants with which foreign antigen must be associated for recognition by T lymphocytes [5]. There is in addition a significant elevation of intercellular adhesion molecule-1 (ICAM-1) and B7 expression; molecules required, respectively, for the interaction of DC with T lymphocytes and for the antigen-driven stimulation of T cell responses [6,7]. In summary, the initiation of cutaneous immune responses to chemical allergens is dependent upon the migration and functional maturation of LC. These processes are effected by cytokines that are produced locally.

3. Epidermal cytokines

Both keratinocytes and epidermal LC are sources of cytokines. Some of these are produced constitutively, while for others an external or internal (autocrine or paracrine) stimulus is required. Among the cytokines produced by keratinocytes are interleukins 1α, 6, 7, 8, 10 and 12 (IL-1α, IL-6, IL-7, IL-8, IL-10 and IL-12), granulocyte/macrophage colony-stimulating factor (GM-CSF) and other hemopoietic growth factors, tumor necrosis factor α (TNF-α), transforming growth factors α and β (TGF-α and TGF-β) and macrophage inflammatory protein 2 (MIP-2). LC are a source of interleukin 1β (IL-1β), IL-6, IL-12, TGF-β, macrophage inflammatory protein 1α (MIP-1α) and MIP-2 [8,9]. By analogy with in vitro studies it is believed that the functional maturation of LC and the acquisition of immunostimulatory potential are mediated by GM-CSF acting in concert with IL-1 and possibly other epidermal cytokines [10]. Another cytokine stimulates the migration of LC. It has been shown that the intradermal injection of mice with homologous recombinant TNF-α causes the migration of LC from the epidermis and the accumulation of DC in regional lymph nodes [11,12]. Systemic treatment of mice with neutralizing anti-TNF-α antibody inhibits almost completely the arrival of DC in draining lymph nodes following skin sensitization [13]. The induction of sensitization therefore requires the action of certain epidermal cytokines that are induced or upregulated following skin contact with chemical allergen. Skin cytokines may serve also to determine the characteristics of cutaneous immune responses to sensitizing chemicals.

In both man and mouse there exists functional heterogeneity among T helper (Th) cells. Two main populations, designated Th1 and Th2, have been identified that develop during the evolution of immune responses. These cells differ with respect to the cytokines they elaborate. Th2 cells produce, among others, interleukin 4 (IL-4), a cytokine required for the initiation and maintenance of IgE production, this being the class of antibody that effects respiratory sensitization [14]. In contrast, Th1 cells mediate delayed-type hypersensitivity reactions including contact hypersensitivity [15]. A product of Th1 cells, interferon γ (IFN-γ), antagonizes IgE responses [16]. It is clear, therefore, that the characteristics of immune responses to chemical sensitizers, and the type of allergic hypersensitivity reaction that will develop subsequently, are to an important extent dependent upon the quality of induced Th cell activation. It has been shown in mice that chemicals known to cause allergic sensitization of the respiratory tract and occupational asthma

induce selective Th2 cell-type responses. Conversely, contact allergens considered not to cause respiratory hypersensitivity provoke immune responses characteristic of Th1 cell activation [17,18].

The development of differentiated Th cell function is determined by the relative availability of cytokines in the immunological microenvironment. In this way the balance between certain epidermal cytokines may influence the quality of cutaneous immune responses. IL-10, a product of keratinocytes (and Th2 cells) and an inhibitor of Th1-type responses, has been shown to influence LC function, to impair the ability of DC to stimulate the production by T lymphocytes of IFN-γ and to inhibit the elicitation of contact hypersensitivity reactions [19–21]. In contrast, IL-12 promotes Th1 cell responses. This cytokine, designated previously natural killer (NK) stimulatory factor, is a product of keratinocytes and has been found recently to be elaborated by DC also. IL-12 stimulates Th1 cell responses directly and also via the stimulation of IFN-γ production by NK cells [22].

The conclusion is that epidermal cytokines are required for the stimulation of cutaneous immune responses to chemical sensitizers and that they may serve also to influence the quality of those responses and the type of allergic disease that develops.

4. Induction and regulation of skin cytokine expression

The initial stimulus for the altered expression of epidermal cytokines undoubtedly derives from local trauma, including encounter on the skin with chemical allergens. It is known for instance that skin sensitization, skin irritation and irradiation with ultraviolet light all result in the increased production by keratinocytes of TNF-α [23,24]. It is clear, however, that epidermal cytokines can be regulated in paracrine fashion. As a consequence, altered expression in some instances may not result directly from an external stimulus, but rather from paracrine stimulation by other cytokines. It was shown by Enk and Katz [23] that very rapidly following skin sensitization of mice there is an increased expression by local LC of mRNA for IL-1β. In subsequent experiments intradermal injection of IL-1β alone was found to initiate many of the changes associated with skin sensitization, including the increased expression of keratinocyte TNF-α [25]. In recent investigations in this laboratory we have considered whether another epidermal cytokine, IL-6, may be regulated in paracrine fashion during the induction phase of contact sensitization. IL-6 is produced constitutively by LC, whereas expression by keratinocytes is inducible [26]. The potential relevance of IL-6 to the development of sensitization is that this cytokine serves as a costimulator of T lymphocyte activation and that its production by draining lymph node cells is induced following topical exposure of mice to chemical allergens [27,28]. An important role for IL-6 in contact sensitization is supported by the observation that the elaboration by lymph node cells of this cytokine correlates closely with the vigor of induced proliferative responses [29].

In preliminary experiments the production of cutaneous TNF-α and IL-6 was measured following topical exposure of mice to a sensitizing concentration of oxazolone, a potent contact allergen. As the results illustrated in Fig. 1 reveal, sensitization resulted in the upregulation of both cytokines. Maximal TNF-α production was observed 2 h following treatment and declined rapidly thereafter. Production of IL-6 was most vigorous at 4 h, but was still elevated significantly 24 h following exposure. We questioned whether the more accelerated induction of TNF-α might influence the subsequent expression of cutaneous IL-6. Consistent with such a role it was found that the intradermal injection of mice with homologous recombinant TNF-α alone caused a significant and rapid (within 2 h) induction of increased IL-6 expression. Identical treatment of mice with bovine serum albumin, the protein in which the recombinant cytokine was suspended, failed to stimulate a similar increase in cutaneous IL-6 production (Fig. 2). Additional experiments were performed to establish whether TNF-α plays a role in the increased expression of IL-6 associ-

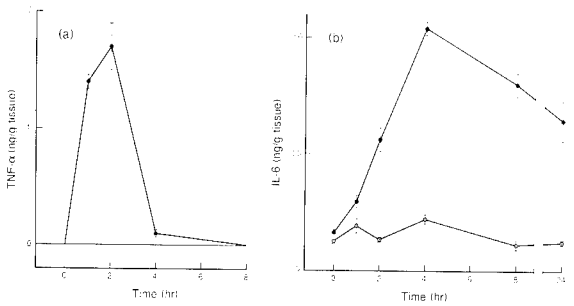


Fig. 1. Influence of topical exposure to oxazolone on cutaneous TNF-α and IL-6 production. Groups of BALB/c strain mice ($n = 4$) were treated on the shaved dorsum with 100 μl 1% oxazolone in 4:1 acetone:olive oil (AOO) (●), or with an equal volume of AOO alone (○). At various times thereafter skin homogenates were prepared from the exposure site and TNF-α (a) and IL-6 (b) content measured using the L929 cytotoxicity assay and an enzyme-linked immunosorbent assay (ELISA), respectively. Results (mean ± S.E.) from a representative experiment are expressed as ng cytokine/g tissue. Limit of detection (horizontal line) = 0.05 ng TNF-α/g tissue.

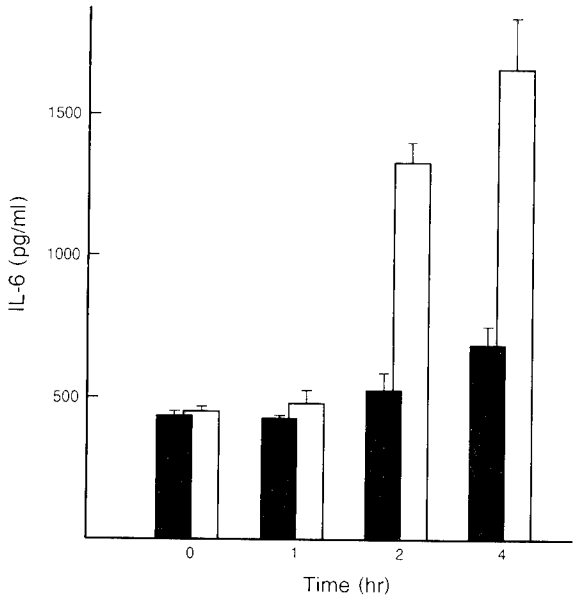


Fig. 2. Influence of TNF-α on cutaneous IL-6 production. Groups of BALB/c strain mice ($n = 4$) received 30 μl intradermal injections into the dorsum of each ear of 50 ng murine recombinant TNF-α (specific activity 2×10^8 U/mg) in 0.1% bovine serum albumin (BSA) (open bars). Control mice received an equal volume of 0.1% BSA alone (hatched bars). At various times thereafter homogenates of the dorsal surface of the ear were prepared and IL-6 content measured by ELISA. Results (mean ± S.E.) from a single representative experiment are recorded as pg/ml IL-6.

ated with skin sensitization. Groups of mice were treated i.p. with a neutralizing anti-TNF-α antibody, or with a control serum, 90 min prior to sensitization with oxazolone. Mice that had received antibody displayed a significant, but incomplete, inhibition of oxazolone-induced IL-6 production (Fig. 3).

The conclusion drawn is that upregulation of IL-6 expression following skin sensitization is in part attributable to the paracrine action of keratinocyte-derived TNF-α. The identity of the cells induced by TNF-α to synthesize IL-6 is of interest. LC have been shown to express this cytokine constitutively [26] and the available evidence suggests that the source of IL-6 in allergen-activated draining lymph nodes is the DC induced to accumulate there. It may be therefore that TNF-α acts directly on LC to upregulate their expression of IL-6. The data illustrated in Fig. 3 reveal that not all cutaneous IL-6 production associated with skin sensitization is dependent upon the availability of TNF-α. One proposal is that increased total cutaneous IL-6 is attributable to both an upregulation of synthesis by LC stimulated in paracrine fashion by TNF-α and an induction of keratinocyte production due to other signals or secondary to a

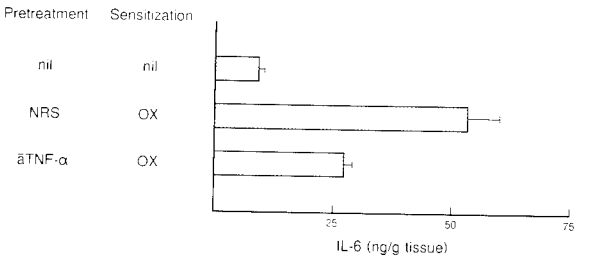


Fig. 3. Influence of anti-TNF-α antibody treatment on oxazolone-induced cutaneous IL-6 production. Groups of BALB/c strain mice ($n = 5$) received a single 100 μl i.p. injection of neutralizing rabbit anti-mouse TNF-α antiserum diluted 1:5 in saline. Control mice received an equal volume of normal rabbit serum (NRS) diluted to an identical extent. Ninety min later mice were treated on the shaved dorsum with 100 μl 1% oxazolone (OX) in AOO. Four h following sensitization skin homogenates were prepared from the exposure site of treated animals and from naive (untreated) controls and IL-6 content measured by ELISA. Results (mean ± S.E.) of a single representative experiment are expressed as ng IL-6/g tissue.

direct affect of the chemical. Certainly the view is that TNF-α is able to interact directly with LC via TNF-R2 (the species-specific 75-kDa membrane receptor for TNF-α) [11,12] and this is consistent with an induction by this cytokine of elevated IL-6 expression by LC.

The above observations can be formulated into a generalized hypothetical scheme for cytokine induction during the initiation phase of skin sensitization. An early event, resulting possibly from the direct interaction of chemical allergen with LC, is the increased production of IL-1β. This cytokine acts locally to effect a number of changes including the induction of TNF-α synthesis by keratinocytes. TNF-α serves in turn to stimulate the migration of LC from the epidermis and to upregulate the expression by these cells of IL-6. The result is that following skin sensitization, LC that have been stimulated to produce increased levels of the costimulatory molecules IL-1β and IL-6 transport antigen in an immunogenic form from the skin to regional lymph nodes. Interference with this network of interactions will impair or inhibit the induction of contact sensitization.

5. Concluding comments

The induction of allergic responses to chemical sensitizers encountered on the skin is dependent upon the action of cytokines produced locally. As described above, epidermal cytokines provide the signal for LC migration from the skin to draining lymph nodes and also mediate the changes in LC phenotype necessary for effective presentation of the inducing allergen to responsive T lymphocytes. Altered or upregulated epidermal cytokine expression is stimulated rapidly following exposure to chemical allergens and there is evidence that paracrine and autocrine processes are important for the induction and regulation of at least some of the cytokines associated with topical sensitization. The relative availability of skin cytokines may serve also to influence the quality of immune responses provoked by chemical sensitizers and thereby the characteristics of allergic disease.

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The use of physiologically-based pharmacokinetic/pharmacodynamic dosimetry models for chemical mixtures

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Abstract

Human exposure to chemicals is rarely, if ever, limited to a single chemical. Therefore, it is essential that we consider multiple chemical effects and interactions in our risk assessment process. However, with the almost infinitely large number of chemical mixtures in the environment, systematic studies of the toxicology of these chemical mixtures with conventional methodologies and approaches are impossible because of the immense resources and unrealistically long durations required. Thus, the development of 'Predictive and Alternative Toxicology' is imperative. At Colorado State University (CSU), our research effort is entirely devoted to this challenge. In order to have a reasonable chance to deal with the complex issue of toxicology of chemical mixtures, we believe that the following concepts must be considered: (1) the utilization of computer; (2) the exploitation of mathematical/statistical methodologies; (3) developing very focused, mechanistically based, and short-term toxicology studies; (4) coupling computer/mathematical modeling with mechanistically-based toxicology. Our strategy is therefore the utilization of physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling, coupled with very focused, model-directed toxicology experiments as well as other statistical/mathematical methodologies such as Monte Carlo simulation, isobolographic analysis, and response surface methodology. We believe that 'Predictive and Alternative Toxicology' in terms of tissue dosimetry at the pharmacokinetic and pharmacodynamic levels is achievable with simple and complex but chemically defined mixtures. In this presentation, we describe two ongoing research projects as an illustration of our 'Bottom-Up' and 'Top-Down' approaches for handling the chemical mixtures: (1) PBPK/PD modeling of toxicologic interactions between Kepone and carbon tetrachloride (CCl_4) and the coupling of Monte Carlo simulation for the prediction of acute toxicity; (2) the conceptual development of PBPK/PD modeling for a more complex chemical mixture of seven groundwater contaminants from hazardous waste sites and the consideration of subfractionation of this chemical mixture.

Keywords: Chemical mixtures; PBPK/PD modeling; Pharmacokinetics; Pharmacodynamics

1. Introduction

What is a chemical mixture? The correct answer is that almost everything around us in the

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environment is a chemical mixture: a breakfast with pancakes, raspberries, orange juice, and coffee; a hamburger with lettuce and tomato; a gourmet dinner of crawfish, asparagus and wine; the suits and dresses we wear; the cosmetics, toiletries and medicines we use, etc. Even our own body is a chemical mixture. Considering all the above then, there is really no such a thing as 'single chemical exposure' in our life.

In contrast with this reality, however, about 95% of the toxicology studies conducted to date have been with single chemicals [1,2]. This represents a very slanted distribution of research resources. From a different perspective, Calabrese [3] insightfully concluded from the outcomes of a number of conferences and workshops held in the 1980s, many with participation of Blue Ribbon Panels of Experts:

"... a careful reading of many of the proceedings from conferences, workshops, and reports of expert committees reveals a repetitious restatement of the obvious: for example, humans are not exposed to single agents; the environment provides exposure to a complex daily mixture of agents; health standards have long ignored the issue of multiple exposures; and this should be an area of high priority..."

"... predictive systems are desperately needed since it is impossible to study all interactions – elementary statistical analysis clearly illustrates the folly of such an exercise..."

Systematic toxicity testing of chemical mixtures in the environment or workplace using conventional toxicology methodologies is highly impractical because of the immense numbers of mixtures involved. For example, a chemical mixture with 25 component chemicals has $2^{[25]} - 1$ or 33 554 431 combinations (i.e. one chemical at a time, any two chemicals in combination, any three in combination, etc.) [1,2]. Furthermore, this huge number of combinations is just for one concentration per chemical. From a different perspective, there are about 600 000 chemicals being used in our society [4]. Just considering binary chemical mixtures, this means that there could be $600\,000 \times 599\,999/2 = 359\,999\,400\,000$ pairs of chemicals. Assuming that only 0.001% of these pairs of chemicals act synergistically or have other toxicologic interactions, there would still be 3 599 994 binary chemical mixtures possessing toxicologic interactions. Further, toxicologic interactions undoubtedly exist among chemical mixtures with three or more component chemicals; the number of possible combinations for these latter mixtures is almost infinite. These are astronomically large numbers with respect to systematic toxicity testing.

2. Approaches for chemical mixtures

Given the above discussion, it is obviously impossible to rely on conventional toxicity testing methodologies to deal with chemical mixtures. Thus, we must use and integrate: (1) computational technology; (2) focused, mechanistically-based, short-term toxicology studies; (3) mathematical/statistical modeling.

Can 'Predictive and Alternative Toxicology' be developed for chemical mixtures using physiologically-based pharmacokinetics/pharmacodynamics (PBPK/PD) coupled with statistical/mathematical modeling? In our opinion, the answer to this question is yes. Because the toxicity produced by xenobiotics in the body is mediated by interactions between the chemicals and their metabolites and biological molecules or structures [5], understanding pharmacokinetics and pharmacodynamics of xenobiotics is therefore a necessity in toxicology. With the advent of PBPK/PD and computer modeling, correlation of quantitative and temporal descriptions of xenobiotic concentrations at target tissues or organs with specific toxicities becomes an attainable reality. By linking the interactive chemical components in a chemical mixture at the level of pharmacokinetic and/or pharmacodynamic modeling, we believe that it is possible to deal with the health effects, collectively, of the chemical mixture of interest.

We propose the 'Bottom-Up' and 'Top-Down' approaches for reaching the ultimate goal of predictive and alternative toxicology for chemical mixtures. Using examples, we explain, briefly, these two approaches below.

2.1. Bottom-up approach

The 'Bottom-Up' approach refers to systematic toxicologic interaction studies starting with binary chemical mixtures based on toxic mechanism(s). Using PBPK/PD modeling as a guide, a third, fourth, etc. chemical is then added based on mechanistic considerations. In this way, we build up the chemical mixture as well as the interlinkage of PBPK/PD modeling of all the components of the chemical mixture. Ultimately, the integrated PBPK/PD model would encompass all the toxicologic interactions in the chemical mixture and it would be able to predict toxicities for the entire mixture. For instance, we have already studied the toxicologic interactions with respect to impairment of liver regeneration by Kepone in the hepatotoxicity of CCl_4 by coupling experimental toxicology, PBPK/PD modeling, and Monte Carlo simulation [6,7]. Since Kepone pretreatment is a prerequisite for this toxicologic interaction, we must hold Kepone as a constant component in all mixtures. Thus, with the addition of two new, known hepatotoxins, 1,1,2,2-tetrachloroethane (1,1,2,2-TE) and hexachloro-1,3-butadiene (HCBD), two new binary chemical mixtures are formed (i.e. Kepone + 1,1,2,2-TE; Kepone + HCBD). Modeling and experimental toxicology results may be obtained on these binary chemical mixtures. Subsequently, we may form the three-component chemical mixtures (i.e. Kepone + CCl_4 + 1,1,2,2-TE; Kepone + CCl_4 + HCBD; Kepone + 1,1,2,2-TE + HCBD), and the four-component chemical mixture (i.e. Kepone + CCl_4 + 1,1,2,2-TE + HCBD). Of course, modeling and experimental toxicology results will be obtained on these chemical mixtures as well. In this manner, more and more complicated chemical mixtures are built up based on mechanisms of toxicity.

As a glimpse of the possible utility of this type of approach, we discuss the findings of PBPK/PD modeling of a binary chemical mixture (Kepone and CCl_4) based on mechanisms of toxicity of interactions and the application of computer technology in acute toxicity studies.

The discussion follows the order of: (1) background toxicology information on Kepone and CCl_4 singly and in combination; (2) our effort on PBPK/PD modeling and model validation using published data; (3) the coupling of PBPK/PD model with Monte Carlo simulation and the prediction of acute toxicity (i.e. mortality), based on pharmacodynamics of hepatotoxicity, in CCl_4-dosed rats with or without pretreatment of dietary Kepone; (4) comparison of computer-predicted results and observed data from experiments conducted in our laboratory.

CCl_4 is a well-known hepatotoxin [8]. Following free radical formation through the P450 enzyme system, the toxicity of CCl_4 can be an accumulation of lipids (steatosis, fatty liver) and degenerative processes leading to cell death (necrosis) [8]. Kepone (also known as chlordecone) is found in the environment as a result of photolytic oxidation of Mirex, a pesticide used for the control of fire ants, or as a pollutant from careless and irresponsible discharge [9]. At relatively low levels (e.g. 10 ppm in the diet), even repeated dosing of Kepone in the diet up to 15 days caused no apparent toxicity to the liver [10].

The toxicologic interaction between Kepone and CCl_4 was reported by Curtis et al. [11]. They illustrated that a 15-day dietary exposure of male rats to Kepone at 10 ppm, an environmentally realistic level of contamination, markedly enhanced liver toxicity produced by an intraperitoneal (i.p.) injection of a marginally toxic dose of CCl_4 (100 μl/kg). This toxicologic interaction is unique in that: (1) unlike many other toxicologic interaction studies which were usually dealing with acute toxicity at very high doses, Kepone in this instance is administered at a very low environmental level; (2) CCl_4 is also dosed at a marginally toxic level; (3) the magnitude of toxicologic interaction is very large. Based on administered dose, the enhancement of CCl_4 lethality is about 67-fold. The mechanism of this toxicologic interaction was elucidated to be the obstruction by Kepone of the liver's regeneration process [12–14].

We chose this binary interaction as a model system to develop our 'Bottom-Up' approach for chemical mixtures. As shown in Fig. 1, the pharmacokinetic portion of the PBPK/PD model was an adaptation of the PBPK model of Paus-

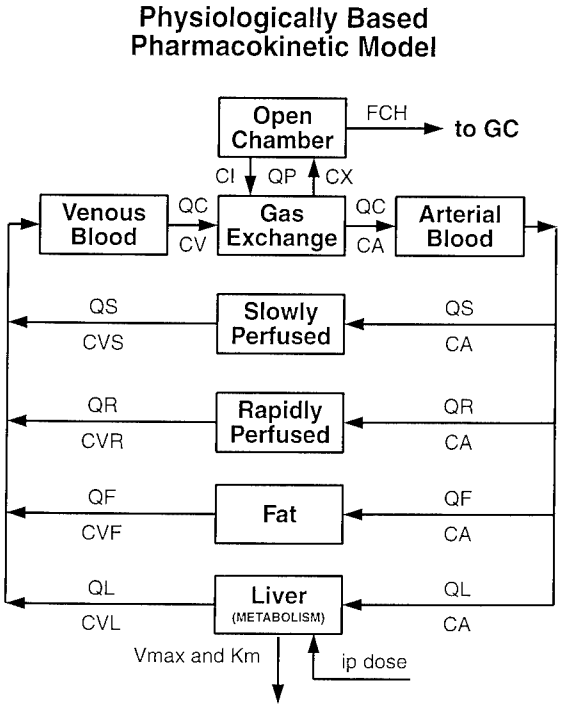


Fig. 1. A PBPK model for CCl_4 adapted from Paustenbach et al. [15]. CI and CX are concentrations of CCl_4 in the inhaled (thus chamber concentration) and exhaled breath; CV and CA represent venous and arterial blood concentrations of CCl_4; Q depicts blood flow rate. S, R, F, L refer to slowly perfused, rapidly perfused, fat, and liver compartments, respectively; Vmax and Km are in vivo hybrid constants representing maximal velocity and affinity constants for enzyme systems involved in the metabolism of CCl_4. (After El-Masri et al. [6].)

tenbach et al. [15]. Initial verification (Fig. 2) of this PBPK model was carried out by using data from exhaled breath analyses from CCl_4-treated rats in our laboratory [7]. This PBPK model was then linked with a PBPD model (Fig. 3), developed in our laboratory based on the latest mechanism of toxicologic interaction between Kepone and CCl_4. By incorporating cell birth/death processes into the PBPK/PD model, time course computer simulations of mitotic, injured, and pyknotic cells after treatment with CCl_4 alone or in combination with Kepone (10 ppm in the diet for 15-day pretreatment) were carried out. Verification of the PBPK/PD model was

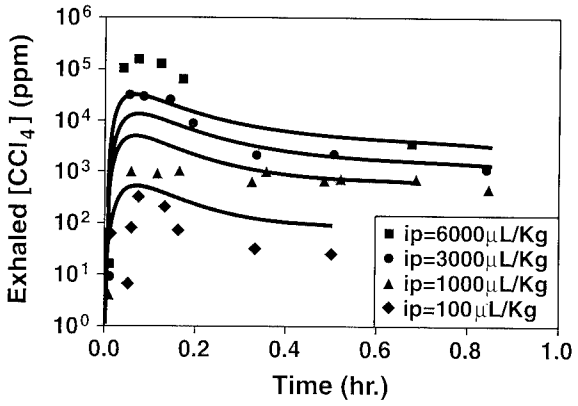


Fig. 2. The PBPK/PD model predictions of the concentrations of CCl_4 in the exhale breath (symbols) of CCl_4-treated rats for different i.p. injections. The lines are the model predictions. (After El-Masri et al. [6].)

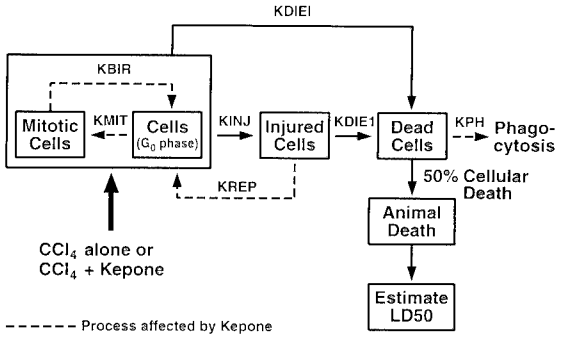


Fig. 3. A PBPD model for toxicologic interactions between Kepone and CCl_4. This depicts the schematic of pharmacodynamic effects of CCl_4 on cellular injury and death. The dashed lines depicts the processes that are affected by the presence of Kepone. When cells are exposed to the reactive metabolites of CCl_4, their inherent death rate is influenced by two mechanisms. A major mechanism of cellular injury leading to death is through lipid accumulation which is illustrated here as the formation of injured cells and dead cells via two rate constants KINJ and KDIE1. For simplicity, all other causes of cell death including natural cell death and other CCl_4-related toxicities are lumped together into a hybrid constant KDIEI as a second mechanism. The injured cells can either be repaired back to viable cells or continue to die. All dead cells, whether induced to die or injured to death, are removed from the liver by phagocytosis. Additionally, the PBPD model considers the effects of CCl_4, alone or in combination with Kepone, on cellular mitotic and birth rates. (After El-Masri et al. [6].)

carried out by comparing simulation results with existing time course data in the literature [16] as shown in Figs. 4 and 5.

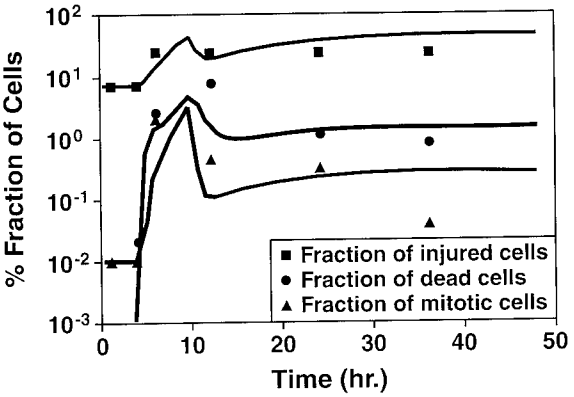


Fig. 4. The PBPK/PD model predictions of the pyknotic, injured and mitotic cells from rats exposed to CCl_4 only. The experimental data were obtained from Lockard et al. [16]. The model predictions are given by the solid lines. (After El-Masri et al. [6].)

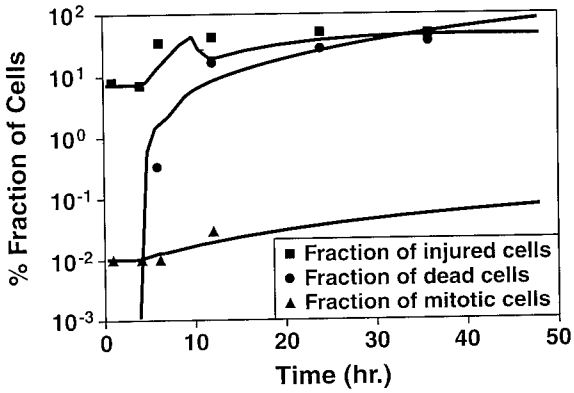


Fig. 5. The PBPK/PD model description of the pyknotic, injured and mitotic cells from rats exposed to CCl_4 and Kepone pretreatment. The experimental data were obtained form Lockard et al. [16]. The model predictions are given by the solid lines. (After El-Masri et al. [6].)

To work toward the goal of 'Predictive and Alternative Toxicology,' this PBPK/PD model was coupled with Monte Carlo simulation to predict the acute lethality of CCl_4 alone and in combination with Kepone (10 ppm in the diet for 15-day pretreatment). In doing so, we were able to conduct acute toxicity studies on computer with a very large sample (i.e. 1000 rats/dose) [7]. As shown in Table 1, these a priori predictions of lethality were in very good agreement with experimentally-derived values except at very high CCl_4 dose levels. In this latter case, the under-prediction of lethality was due to toxicity other than the liver, most likely neurotoxic effects on the central nervous system. Histomorphometric analyses of liver supported this explanation [6,7]. The extent and prevalence of hepatocellular necrosis at 6000 μl/kg was disproportionally small because some of the rats died of CNS effects of CCl_4 before hepatotoxicity could be developed.

2.2. Top-down approach

The 'Top-Down' approach, as the name implies, will start out with a more complex chemical mixture of several to many component chemicals. We use below a chemical mixture of seven groundwater contaminants (arsenic, benzene, chloroform, chromium, lead, phenol, and trichloroethylene) to illustrate the essence of the 'Top-Down' approach. From earlier studies [17,18], we have already obtained interesting preliminary findings on the complete mixture and some of its submixtures. Since there are $2^{[7]} - 1 = 127$ combinations for seven chemicals at only one concentration, systematic toxicity testing on all the combinations is a prohibitively expensive effort. Thus, we tried to minimize experimentation by using educated guesses to set study priorities on those submixtures to be tested. For instance, the initial fractionation into a metal submixture and organic chemical submixture appeared to be a reasonable first step. Because we were interested in finding out the potential promoter activities of this chemical mixture, its submixtures, and components, further subfractionations according to the known carcinogenicity of these chemicals seem to be a reasonable approach as well. In this manner, we conduct experiments on finer and finer submixtures until we get to individual chemicals. As a representative scenario, Fig. 6 illustrates this approach graphically.

Using the 'Top-Down' approach, we will study a total of five chemical mixtures and seven individual chemicals. Although the overall number of combinations for seven chemicals is 127, we believe that a simplified top-down scheme (Fig. 6) based on our knowledge and experience for the seven chemicals would be sufficient to

Table 1
Kepone/carbon tetrachloride-induced mortality by PBPK/PD modeling coupled with Monte Carlo simulation vs. experimentally observed (after El-Masri et al. [6])

Dose given		Model predictions[a]		Observed[b]	
Kepone (ppm)	CCl_4 ($\mu l\ kg^{-1}$)	Dead rats	% Dead	Dead rats	% Dead
0	100	0	0.0	0	0.0
0	1000	1–2	13.2	1	11.1
0	3000	3	32.8	4	44.4
0	6000	4–5	47.8	8	88.8
10	10	0	0.0	0	0.0
10	50	4–5	47.5	4	44.4
10	100	8–9	84.0	8	88.8

[a] Mortalities in 48 h, $n = 9$; Monte Carlo simulation, $n = 1000$.
[b] Actual lethality studies ($n = 9$).

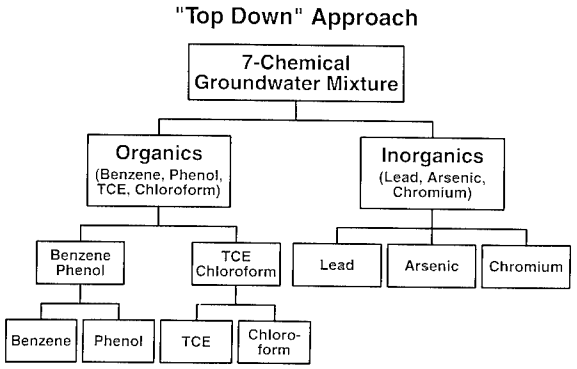


Fig. 6. 'Top-Down' approach to evaluation of chemical interactions in chemical mixtures of groundwater contaminants from Superfund hazardous waste disposal sites. (After El-Masri et al. [20].)

tease out the chemicals responsible for the findings (i.e. increased liver cell proliferation around the hepatic vein, pharmacokinetic, and enzyme kinetic changes) from our earlier studies [17,18].

PBPK/PD modeling of chemical mixtures beyond two components is at an embryonic stage. Therefore, our discussion here is largely conceptual. The simplest and most primitive way is to consider the whole mixture as a single entity. Depending on the endpoint(s) chosen such as liver cell proliferation, enzyme or pharmacokinetic parameter changes, we may consider, in the modeling process, the effects of this entire chemical mixture upon that specific endpoint. There are some concerns about this approach and they are discussed elsewhere [19,20].

The most complicated, and thus most refined, way is to have a PBPK/PD model for each of the chemical components in the chemical mixture. These models are then linked at pharmacokinetic and/or pharmacodynamic level(s) to include all known toxicologic interactions. Even though this may require very complex modeling and computer simulation, the current capability of computational technology should permit us to do so. In between the above two ways is a compromise approach. Here the chemical engineering concept of 'lumping analysis' [21] may be applied to 'lump' certain chemicals into a group as an entity. Considering the successful application of this technique in chemical engineering processes, it is reasonable to assume that the application of 'lumping analysis' for PBPK/PD modeling of chemical mixture toxicology is possible.

3. Future directions and refinement of PBPK/PD modeling

The above experiments and approaches represent the first step in our development of 'Predictive and Alternative Toxicology.' There is definitely room for improvement. Presently, two aspects are being explored. First, to improve the PBPK/PD model for Kepone and CCl_4 interaction, we are incorporating: (1) a PBPK model for Kepone to account for the pharmacokinetics of Kepone which will be linked with the CCl_4

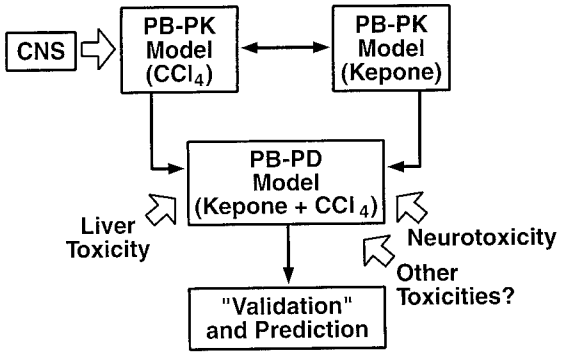


Fig. 7. Further refinement of the PBPK/PD Model for Kepone and CCl_4: addition of other toxicity endpoints to form an 'Integrated Toxicology Model.' (After El-Masri et al. [20].)

model; (2) because of the obvious limitation of a PBPK/PD model which centers exclusively on pharmacodynamics of liver toxicity, we should consider the concept of an 'Integrated Toxicology Model' in our PBPK/PD modeling to account for different toxicologic endpoints under the conditions of a wider dosing regimen. Fig. 7 is a graphical illustration of such a concept for Kepone and CCl_4. Second, PBPK/PD modeling on more complex chemical mixtures deserves a great deal of effort. This is an area in which the future of toxicology lies.

4. Discussion and perspectives

Our research effort on PBPK/PD modeling with chemical mixtures aims at developing 'Predictive and Alternative Toxicology.' By 'Predictive Toxicology,' we are referring to tissue dosimetry at the pharmacokinetic and pharmacodynamic levels. By 'Alternative Toxicology,' we are working toward minimizing animal experimentation, as illustrated in the example given on Monte Carlo simulation coupled with PBPK/PD modeling of Kepone/CCl_4 interactions. The application of PBPK/PD to risk assessment of chemical mixtures may have several advantages: (1) The incorporation of mechanistic information on toxicologic interactions; (2) It conserves resources and it reduces animal killing and suffering in the Hazard Identification step; (3) Reducing the necessity of using large uncertainty factors. Thus, PBPK/PD modeling will provide more realism into the risk assessment process. Of course, one must be aware of the fact that PBPK/PD modeling has its own intrinsic 'uncertainties'; therefore, as much as practicable, any PBPK/PD model must be rigorously validated with experimental results before 'Predictive Toxicology' so derived becomes meaningful.

The linkage of two of the most challenging areas in toxicology today: (a) PBPK/PD and statistical/mathematical modeling; and (b) experimental toxicology of chemical mixtures will have immense potential in application to risk assessment to chemical mixtures. Fig. 8 is our strategy for 'Predictive and Alternative Toxicology' for chemical mixtures and the development of 'Innovative Risk Assessment Methodologies for Chemical Mixtures.' We are attempting to couple PBPK/PD and other experimental toxicology with isobolographic analysis and/or response surface methodology for the modeling and analysis of toxicologic interactions. With the aid of techniques such as Monte Carlo simulation, we may better predict tissue dosimetry at the pharmacokinetic and pharmacodynamic levels. Using such values as benchmark doses,

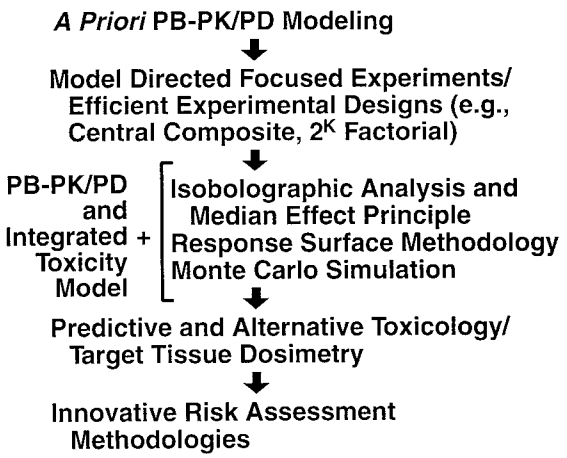


Fig. 8. Our proposed strategy/approach to develop 'Predictive and Alternative Toxicology' and formulate 'Innovative Risk Assessment Methodology' for chemical mixtures. (Modified from El-Masri et al. [20].)

human risk assessment of chemical mixtures may be carried out with less uncertainty.

Acknowledgements

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Toxicity studies in rats of simple mixtures of chemicals with the same or different target organs

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Abstract

Mixtures of chemicals with different target organs or the same target organ but different target sites or different modes of action did not appear to be distinctly more hazardous than the individual chemicals, provided the dose level of each chemical in the mixture did not exceed its own 'No-Observed-Adverse-Effect Level'. Clearly, for such mixtures and exposure conditions the additivity assumption did not hold. However, the additivity rule appeared to be applicable to mixtures of chemicals with the same target organ and the same mechanism of action or receptor. Fractional 2-factorial study designs were found to be promising tools for examining possible combined actions or interactions of chemicals in a mixture.

Keywords: Simple mixtures; Rats; Similar/different target organs/mode of action

1. Introduction

Chemical mixtures are characteristic of life, and humans are concurrently or sequentially exposed to a huge number of chemicals [1]. This reality indicates the necessity of exposure assessment, hazard identification and risk assessment of chemical mixtures [2]. However, it has been estimated that to date about 95% of the resources in toxicology are devoted to single chemicals [3]. Fortunately, the interest of scientists and regulators in the toxicology of mixtures is growing.

National and international organizations involved in standard setting usually suggest the use of simple dose or response addition models for assessing the hazard of a chemical mixture, ignoring the mode of action of the chemicals. Such an approach is invalid for mixtures for which the additivity assumption does not hold, and will lead to overestimation of the hazard in case of chemicals with dissimilar (independent) joint action and to underestimation of the hazard in case of chemicals with potentiating interaction.

One of the major aims of our Institute's research programme on the toxicology of mixtures is to test the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern [4]. To this end we carried out a

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series of short-term oral and inhalation toxicity studies in rats with chemicals with the same or different target organs and with similar or dissimilar mechanism of action. The present paper describes studies with mixtures of chemicals with:
(1) different target organs and/or different modes of action;
(2) same target organ, different modes of action;
(3) same target organ, different target sites; and
(4) same target organ (kidneys or nose), similar mode of action.

2. Mixtures of chemicals with different target organs and/or different modes of action

We conducted two 4-week toxicity studies in rats with combinations of 8 or 9 different chemicals, respectively [5,6]. Chemicals, dose levels and major target (organ) are presented in Tables 1 and 2. In each study an appropriate control group was included. The major purpose of the studies was to determine whether simultaneous administration of the chemicals at doses equal to the 'No-Observed-Adverse-Effect Level' (NOAEL) of each of the individual chemicals would result in a NOAEL or an adverse-effect level for the mixture.

As compared with the adverse effects seen at the 'Lowest-Observed-Adverse-Effect Level' (LOAEL) of the individual chemicals, both more severe and less severe effects were observed following simultaneous administration at the LOAEL, indicating addition, potentiating interaction and antagonistic interaction at this dose

Table 1
Concentrations of test chemicals (and their major target) in food or drinking water for various dose groups in a 4-week oral toxicity study of a combination of 8 chemicals in rats [5]

Chemical	Concentration (ppm) in food or drinking water[a] given to the group indicated by:				Major target at LOAEL
	NOAEL/10	NOAEL/3	NOAEL	LOAEL	
KNO_2	10	33	100	300	Adrenals
Stannous chloride	100	330	1000	3000	Body weight, haemoglobin
$Na_2S_2O_5$	500	1670	5000	20 000	Red blood cell, stomach
Metaldehyde	20	70	200	1000	Liver
Loperamide	0.5	1.7	5	25	Body weight
Mirex	0.5	1.7	5	80	Body weight, liver
Lysinoalanine	3	10	30	100	Kidneys
DOTC	0.6	2	6	30	Thymus

[a] Only KNO_2 was administered in the drinking water.
DOTC, di-*n*-octyltin dichloride.

Table 2
Chemicals and their major target organs as well as the exposure levels (mg/kg diet or ppm in air) used in a 4-week toxicity study in male rats [6]

Chemical	LOAEL	NOAEL	1/3 NOAEL	Target organ
Aspirin	5000	1000	330	Liver, stomach
Cadmium chloride	50	10	3	Red blood cell, liver
Stannous chloride	3000	800	260	Red blood cell
Loperamide	30	6	2	Liver
Spermine	2000	400	130	Heart, liver
BHA	3000	1000	330	Stomach
DEHP	1000	200	65	Liver
Dichloromethane	500	100	30	Blood
Formaldehyde	3	1	0.3	Nose

BHA, butyl hydroxyanisol; DEHP, di(2-ethylhexyl)phthalate.

level. Except for some minor changes in a few parameters, no treatment-related adverse effects were observed at the NOAEL of the mixtures. Both studies allowed the overall conclusion that exposure to the mixture of chemicals did not constitute an evidently increased hazard, provided the dose level of each chemical in the mixture is a NOAEL [4,7].

The results of each of the above studies also suggested, though in an indirect manner because they were designated for a different purpose, that in case of a quantitatively arbitrary composition of these mixtures the NOAEL of the mixtures would largely, if not exclusively, be determined by the NOAEL of the chemical with the smallest margin between its actual level in the mixture and its true no-adverse-effect level, assuming that the actual level of each of the chemicals in the mixture is lower than its true no-adverse-effect level. Or expressed in terms of health risk, the risk of the mixtures studied is largely, if not entirely, determined by the risk associated with the chemical in the mixture with the highest risk quotient ('exposure level' divided by 'toxicity level'), provided the risk quotient of each of the other chemicals in the mixture does not exceed unity. (We suggest to designate the chemical in a mixture with the highest risk quotient as the 'most risky chemical' of the mixture [7].)

3. A mixture of chemicals with the same target organ but with different modes of action

We carried out a 4-week oral toxicity study in rats with a mixture of 4 nephrotoxicants administered in the diet [8]. The selected chemicals, the dose levels and the modes of action are presented in Table 3. The aim of the study was to find out whether simultaneous administration of 4 nephrotoxicants at a dose equal to their individual 'No-Observed-Nephrotoxic-Effect Level' (NONEL) would result in an adverse-effect level for the combination. A large number of parameters was used to assess the toxicity of the nephrotoxicants.

The individual chemicals caused slight growth depression in males at the 'Lowest-Observed-Nephrotoxic-Effect Level' (LONEL) but not at the NONEL, whereas the combination depressed growth very slightly at the NONEL and severely at the LONEL. Males fed the combination showed decreased renal concentrating ability and moderate histopathological changes in the kidneys at the LONEL, and a dose-dependent increase in kidney weight and number of epithelial cells in the urine at the NONEL and the LONEL. No adverse effects attributable to treatment were observed in rats fed the combination at one-quarter of the NONEL. It was concluded that combined exposure to 4 nephrotoxicants at their individual NONEL did not constitute an *obviously* increased hazard, indicating absence of potentiating interaction and most probably also of *clear* additivity [8].

4. Mixtures of chemicals with the same target organ but with different target sites

Formaldehyde, acetaldehyde and acrolein are well-known nasal irritants. To study possible additive or interactive effects on the nasal epithelium we performed 1- and 3-day inhalation studies (6 h/day) with mixtures of these aldehydes, using male Wistar rats and exposure concentrations varying from clearly non-toxic to toxic (Table 4) [9]. A series of biochemical and

Table 3
Dose levels (ppm in the diet) and modes of action of nephrotoxicants used in a 4-week feeding study in rats [8]

Nephrotoxicant	NONEL/4	NONEL	LONEL	Mode of action
Lysinoalanine	7.5	30	240	Metal ion chelator
Mercuric chloride	3.75	15	120	Mitochondrial dysfunction
Hexachloro-1,3-butadiene	5	20	100	β-Lyase mediated activation
d-Limonene	125	500	4000	$\alpha_{2\mu}$-Globulin accumulation

Table 4
Exposure concentrations (ppm) of formaldehyde, acetaldehyde and acrolein used in 1- and 3-day (6 h/day) inhalation toxicity studies in rats [9]

Group code[a]	Time of exposure (days)	Formaldehyde	Acetaldehyde	Acrolein
Control	1 and 3			
Form/L	3	1.0[b]		
Form/H	1 and 3	3.2[c]		
Acet/L	1 and 3		750[b]	
Acet/H	1 and 3		1500[c]	
Acro/L	3			0.25[b]
Acro/H	1 and 3			0.67[c]
Mix 1	3	1.0[b]		0.25[b]
Mix 2	1 and 3	1.0[b]	750[b]	0.25[b]
Mix 3	1 and 3	3.2[c]	1500[c]	0.67[c]

[a] Form, formaldehyde; Acet, acetaldehyde; Acro, acrolein; L, low; H, high.
[b] NOAEL (in fact 0.25 ppm acrolein induced some very subtle changes).
[c] LOAEL.

morphological parameters was used to assess the nasal toxicity.

Effects were primarily observed after 3 days of exposure. Cell proliferation and histopathological changes of the nasal epithelium induced by Mix 3 (mixture of the aldehydes at their LOAELs; Table 4) appeared to be more severe and more extensive both in the respiratory and olfactory part of the nose than those observed after exposure to the individual aldehydes at comparable exposure levels. This interpretation of increased nasal toxicity of the mixture compared to that of the individual chemicals might be an overinterpretation because the increased nasal toxicity may (partly) be due to differences in degree and site of deposition of the aldehydes in the mixture caused by differences in airflow pattern between rats exposed to single aldehydes and rats exposed to mixtures of aldehydes. However, neither effect addition nor potentiating interactions occurred at NOAELs (Mix 1 or Mix 2; Table 4). Overall, the findings in these studies with nasal cytotoxicants suggest that, *for NOAELs*, combined exposure to these aldehydes with the same target organ (nose) and exerting the same type of adverse effect (nasal cytotoxicity), but partly with different target sites (different regions of the nasal mucosa), is not associated with a greater hazard than that associated with exposure to the individual chemicals.

5. Mixture of chemicals with the same target organ and similar mode of action

5.1. Nephrotoxicants

Subsequent to the study on chemicals with the same target organ (kidney) but different mechanisms of action, we conducted a study to test the additivity assumption (dose addition) under conditions of concurrent, repeated exposure to similarly acting nephrotoxicants, at levels slightly below the LONEL of the individual compounds [7]. Tetrachloroethylene, trichloroethylene, hexachloro-1,3-butadiene and 1,1,2-trichloro-3,3,3-trifluoropropene were used as model compounds. Their nephrotoxicity results from initial conjugation to glutathione in the liver, and cysteine conjugate β-lyase-mediated formation of reactive metabolites in the proximal tubular epithelial cells. The compounds were given to female rats by daily oral gavage for 32 days either alone, both at the LONEL and at the NONEL (equivalent to LONEL/4), or in combinations of 4 (at the NONEL and LONEL/2) or 3 (at the LONEL/3) (Table 5).

Relative kidney weight was increased following exposure to the individual compounds at their LONEL and, to about the same extent, following combined exposure at the NONEL (LONEL/4) or LONEL/3. The other end-

Table 5
Treatments, test chemicals and dose levels used in a 32-day oral toxicity study with mixtures of nephrotoxicants in rats [7]

Treatment (doses in mg/kg body weight)	Total dose in toxicity units
Control: corn oil 10 ml/kg	0
Individual compounds at NONEL[a]	
TETRA 600 mg/kg	1/4
TRI 500 mg/kg	1/4
TCTFP 1.5 mg/kg	1/4
HCBD 1 mg/kg	1/4
Individual compounds at LONEL	
TETRA 2400 mg/kg	1
TRI 2000 mg/kg	1
TCTFP 6 mg/kg	1
HCBD 4 mg/kg	1
Combination of all 4 compounds	
At NONEL	1
At LONEL/2	2
Combinations of 3 compounds	
TETRA + TRI + TCTFP at LONEL/3	1
TETRA + TRI + HCBD at LONEL/3	1
TETRA + TCTFP + HCBD at LONEL/3	1
TRI + TCTFP + HCBD at LONEL/3	1

[a] = LONEL/4.
TETRA, tetrachloroethylene; TRI, trichloroethylene; TCTFP, 1,1,2-trichloro-3,3,3-trifluoropropene; HCBD, hexachloro-1,3-butadiene.

points used to assess renal toxicity (namely, histopathology, concentrating ability, urinary excretion of glucose, protein and marker enzymes, and plasma creatinine and urea) were not or only scarcely affected upon combined exposure at the NONEL or LONEL/3. Interpretation hereof is complicated because these endpoints, unlike kidney weight, were not affected at the LONEL of each of the individual compounds. Co-administration at the LONEL/2 resulted in clear nephrotoxicity as indicated by the effects on most of the above endpoints. It is concluded that the renal toxicity, as assessed by the effect on kidney weight, of a mixture of similarly acting nephrotoxicants, at levels slightly below the LONEL of the individual compounds, corresponded to the effect expected on the basis of the additivity assumption [7]. It is self-evident that for this example the 'dose addition' model (simple similar action or similar joint action) represents the basic concept to be used for risk assessment. This model appeared to be applicable to NOAELs (the highest level of each chemical at which no adverse effect was observed), and in all likelihood will also be applicable to low non-toxic-effect levels.

5.2. Sensory irritants

Sensory irritation of formaldehyde, acrolein and acetaldehyde as measured by decrease in breathing frequency (DBF) was studied in male Wistar rats using nose-only exposure [10]. After an acclimatization period of 10 min, groups of 4 rats were exposed to each of the chemicals separately or to mixtures of formaldehyde, acrolein and acetaldehyde for 30 min followed by a 10-min recovery period. The respiratory rate of each rat was measured during the entire test period with 1- or 3-min intervals. The studies with mixtures were conducted at exposure concentrations expected to result in a DBF between 10 and 35% for each of the individual aldehydes. For the mixtures the predicted values were compared with the observed values using both the model for effect addition and the mechanistic model for competitive agonism [11].

The latter model is based on the assumption that the aldehydes show competitive interactions for a common binding site, i.e. the trigeminal nerve receptor. As expected each of the 3 aldehydes appeared to act as sensory irritants as defined by Alarie [12]. With formaldehyde and acrolein desensitization (fast fading of the DBF) occurred whereas with acetaldehyde the breathing frequency gradually decreased with increasing exposure time (up to 30 min). This latter effect might have been caused by either pulmonary irritation or the anaesthetic effect of acetaldehyde. For the mixtures, the observed DBFs were larger than the expected DBFs for each of the individual chemicals, but were less than the sum of these latter DBFs. This less than purely additive effect could be described by a mechanistic model for competitive agonism. The results also showed absence of desensitization with mixtures; in fact, a second, more gradual DBF was observed in most of the rats exposed to mixtures which might have been caused by pulmonary irritation (induced by acetaldehyde).

The results of this study allow the conclusions that (1) sensory irritation in rats exposed to mixtures of irritant aldehydes is more severe than that caused by each of the aldehydes separately, and (2) the DBF caused by mixtures of these aldehydes can be predicted by a mechanistic model for competitive agonism between the 3 chemicals [10].

6. The use of fractional 2-factorial designs

The 4-week toxicity study with a mixture of 9 chemicals with different target organs and/or different modes of action comprised 3 main test groups (plus 1 control group) as shown in Table 2, as well as 16 satellite groups (Table 6) [6]. In the main groups we investigated the net com-

Table 6
Test groups and exposure levels of a 4-week toxicity study with combinations of 9 compounds in male rats [6]

	Formaldehyde (F)	Dichloromethane (M)	Aspirin (A)	$CdCl_2$ (Cd)	$SnCl_2$ (Sn)	Loperamide (L)	Spermine (Sp)	BHA (B)	DEHP (D)
Main groups									
Control	–[a]	–	–	–	–	–	–	–	–
NOAEL/3	+[a]	+	+	+	+	+	–	+	+
NOAEL	+	+	+	+	+	+	+	+	+
LOAEL	+	+	+	+	+	+	+	+	+
Satellite groups[b]									
F	+	–	–	–	–	–	–	–	–
Sn/M/L/A	–	+	+	–	+	+	–	–	–
Cd/M/Sp/A	–	+	+	+	–	–	+	–	–
Sn/Cd/Sp/L/F	+	–	–	+	+	+	+	–	–
B/M/Sp/L	–	+	–	–	–	+	+	+	–
Sn/B/Sp/A/F	+	–	+	–	+	–	+	+	–
Cd/B/L/A/F	+	–	+	+	–	+	–	+	–
Sn/Cd/B/M	–	+	–	+	+	–	–	+	–
D/Sp/L/A	–	–	+	–	–	+	–	–	+
Sn/D/M/Sp/F	+	+	–	–	+	–	+	–	+
Cd/D/M/L/F	+	+	–	+	–	+	–	–	+
Sn/Cd/D/A	–	–	+	+	+	–	–	–	+
B/D/M/A/F	+	+	+	–	–	–	–	+	+
Sn/B/D/L	–	–	–	–	+	+	–	+	+
Cd/B/D/Sp	–	–	–	+	–	–	+	+	+
All LOAEL[c]	+	+	+	+	+	+	+	+	+

Dose levels of LOAEL and NOAEL are given in Table 2. Main groups and satellite groups comprised 8 and 5 animals, respectively.

[a] –, is absent; +, is present.

[b] All chemicals were administered at the LOAEL.

[c] Data of 5 animals of the main study were used for the establishement of the 16th group of the satellite study.

bined effects of all compounds. In the satellite groups the rats were simultaneously exposed to various combinations of several of the 9 compounds. The combinations used comprised a fractional 2-level factorial design with 9 factors (9 chemicals) in 16 experimental groups. In this case we used a 1/32 fraction of a 'complete' study with $2^9 = 512$ possible combinations. The combinations were chosen such that the results would allow for an optimal analysis of effects of the individual chemicals, indicating different types of joint action or interaction between the chemicals. In contrast to the large number of adverse effects observed with the combination at the LOAEL, only very few adverse effects were encountered in the NOAEL-group such as hyperplasia of the nasal respiratory epithelium, hepatocellular hypertrophy, decreased blood triglyceride concentrations, decreased alkaline phosphatase activities and increased (relative) kidney weights. A detailed analysis of the extent of the interactive effects between the individual compounds was possible due to the application of the fractionated design as used in the satellite part of the study. For most of the endpoints, the factorial analysis indeed revealed the main effects of the individual compounds and also interactions (cases of non-additivity) between the chemicals chosen. Despite restrictions and pitfalls that are associated with the use of fractionated factorial designs, the present study showed the usefulness of these designs to study the joint adverse effects of defined chemical mixtures. Because of the huge number of endpoints studied (about 80 parameters were used) that were not affected at the NOAEL of the mixture, it was concluded that simultaneous exposure to these 9 chemicals did not constitute an evidently increased hazard compared to exposure to each of the chemicals separately, provided the exposure level of each chemical in the mixture is at most similar to or lower than its own NOAEL [6].

7. Concluding remarks

To successfully study and to understand the toxicology of chemical mixtures it is essential to be familiar with the basic concepts of combined actions and interactions of chemicals in a mixture. It is also crucial to understand the principles of statistical designs that allow identification of combined actions or interactions of chemicals, using a manageable number of test groups [6].

A number of studies reviewed in this paper demonstrate that exposure to mixtures of chemicals with different modes of action (simple dissimilar action) does not constitute an evidently increased hazard compared to that of exposure to the individual chemicals, provided the exposure level of the chemicals in the mixture is at most equal to or slightly lower than their own NOAEL. The health risk of such mixtures is entirely determined by the health risk associated with the 'most risky chemical' in the mixture, provided the risk quotients of the other chemicals in the mixture are at most equal to unity [7]. This also means that exposure to such mixtures is of no health concern when all chemicals in the mixtures occur at (low) non-toxic-effect levels.

The studies with mixtures of chlorinated nephrotoxicants with similar mode of action demonstrated the applicability of the dose addition concept to these types of mixture for assessing their possible health risk. Hopefully, this kind of evidence prevents the use of this concept in risk assessment of chemical mixtures for which the additivity assumption is invalid, because misuse can easily lead to overestimation (in case of chemicals with dissimilar action) or underestimation (in case of potentiating interaction) of the health risk associated with such mixtures [7].

The results of the inhalation studies in rats with mixtures of formaldehyde, acetaldehyde and acrolein showed that the type of combined or interactive effects of the mixtures on the nasal mucosa found at clearly-cytotoxic-effect levels did not predict very well the kind of combined action occurring with combinations of the aldehydes at NOAELs of the individual chemicals. Precisely, these and lower exposure levels are where the interest of the risk assessor lies, and where, remarkably enough, toxicological data are often lacking. In combination toxicology much effort is put into elucidating mechanisms of (inter)action at relatively high toxic-effect levels, whereas the transitional area between toxic and

non-toxic levels is virtually unexplored. The toxicology of mixtures should focus on this 'twilight zone'.

Sensory irritation of mixtures of formaldehyde, acrolein and acetaldehyde as measured by DBF in rats appeared to be more marked than the sensory irritation expected for each of the individual aldehydes but much less marked than the sum of the irritant activities of the individual chemicals. The irritant potencies of the mixtures could be accurately described by a mechanistic model for competitive agonism [10,11].

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Lack of carcinogenicity of pesticide mixtures administered in the diet at acceptable daily intake (ADI) dose levels in rats

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Abstract

Carcinogenic effects of pesticide mixtures were examined with our medium-term carcinogenesis protocols using male F344 rats. In the 8-week liver model, combined dietary administration of 20 pesticides (19 organophosphorus compounds and 1 organochlorine), each at acceptable daily intake (ADI) levels, did not enhance rat liver preneoplastic lesion development initiated by diethylnitrosamine. In contrast, a mixture of 100 times ADI significantly increased the number and area of liver lesions. In the second experiment using a multi-organ carcinogenicity protocol of 28 weeks, mixtures of 40 pesticides (high volume compounds) and 20 pesticides (suspected carcinogens) added to the diet at their respective ADI levels did not enhance carcinogenesis in any organ initiated by 5 different known carcinogens in combination. These results provide support for the safety factor (usually 100) approach presently used for the quantitative hazard evaluation of pesticides.

Keywords: Pesticide; Chemical mixture; Acceptable daily intake (ADI); Carcinogenesis; Medium-term bioassay; Rat

1. Introduction

As possible environmental toxic or carcinogenic agents, agrochemicals deserve particular attention [1,2]. Not only workers in the industry and agricultural fields but also the general population are potentially at risk of exposure to such chemicals in foods. Although the assessment of human cancer risk associated with specified chemical exposure is a complicated scientific endeavor, the WHO Expert Groups on Pesticide Residues and the Food and Agriculture Organization of the United Nations (FAO), which regularly hold joint meetings on Pesticide Residues, have set an acceptable daily intake (ADI) for each pesticide as one approach to quantitative hazard evaluation [3]. The ADI levels evaluated by the FAO/WHO have been quoted and used as a basis for further evaluation by many governments in the world including Japan where the values have been slightly modified taking into account additional data.

We have conducted extensive study of the carcinogenic activity of pesticides over the last several years using our medium-term bioassay systems [4–8]. In order to confirm the efficacy of

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this approach we have used ADI values. Since additive or synergistic effects of complex mixtures have become increasingly important for risk estimation in human toxicity [9–11], we tested the possible carcinogenic influence of mixtures of 20 or 40 pesticides given in the diet using the 2 types of our medium-term bioassays for rapid detection of carcinogens [12]. At the present time, all pesticides examined are approved for use in Japan.

2. Materials and methods

Male F344 rats, 6 weeks old, were maintained on powdered rat chow with or without pesticide supplementation. The ADI values used were proposed by the Ministry of Health and Welfare, Japan, with reference to the FAO/WHO reports [3]. Concentrations of pesticides in the diet (weight of chemical/total diet weight $\times 10^6$ or ppm) were calculated based on our previous food intake and body weight data [13]. Food and water were available ad libitum.

2.1. Experiment 1

The pesticides investigated are listed in Table 1 along with the ADI levels, results of the Ames test, and carcinogenicity test results in the literature. Except for endosulfan, all pesticides examined are organophosphorus compounds. Carcinogenicity has been reported for dichlorvos in pancreas (adenoma) and hematopoietic system of rat and forestomach of mouse and for methidathion in the liver of mouse. Although liver carcinogenicity is suspectable, malathion has been reported not to be carcinogenic [see Ref. 13]. Protocol of the medium-term liver bioassay is shown in Fig. 1. The animals were initially given a single i.p. injection of diethylnitrosamine (DEN) at a dose of 200 mg/kg to initiate

Table 1
Organophosphorus pesticides used in the medium-term liver bioassay (Exp. 1)

Chemical	ADI (mg/kg/day)	Concentration in the diet (ppm)	Mutagenicity (Ames test)	Carcinogenicity and target organ (species)
Pesticides (16)				
Acephate	0.03	0.3	+	Liver (mouse)
Chlorpyrifos	0.01	0.1	+	–
Chlorfenvinphos	0.0015	0.015	+	?
Dichlorvos	0.0033	0.033	+	Pancreas (rat), forestomach (mouse)
Dimethoate	0.01	0.1	+	Digestive, lung, mammary gland, ovary (rat and mouse)
Endosulfan[a]	0.006	0.06	+	–
Etrimfos	0.003	0.03	?	?
Fenitrothion	0.005	0.05	+	–
Isoxathion	0.003	0.03	–	?
Malathion	0.02	0.2	–	–
Methidathion	0.001	0.01	?	Liver (mouse)
Pirimiphos-methyl	0.01	0.1	?	?
Prothiophos	0.0015	0.015	–	?
Pyraclofos	0.001	0.01	?	?
Trichlorfon	0.01	0.1	+	–
Vamidothion	0.008	0.08	+	?
Fungicides (3)				
Edifenphos	0.0025	0.025	–	?
Iprobenfos	0.003	0.03	–	?
Tolclofos-methyl	0.064	0.64	?	?
Herbicides (1)				
Butamifos	0.0016	0.016	?	?

[a] Endosulfan is an organochlorine compound.
?: not reported as far as we know.

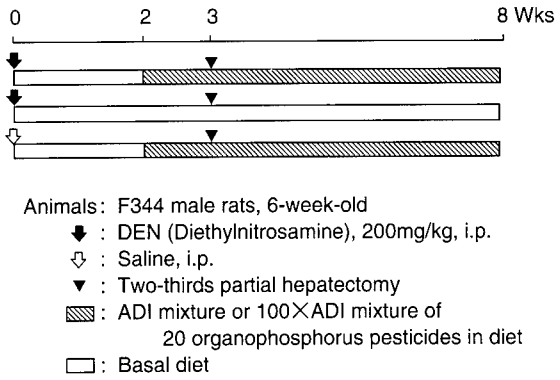


Fig. 1. Experimental protocol of the medium-term liver bioassay.

hepatocarcinogenesis. After a 2-week recovery period, the rats received the pesticides either at ADI (ADI mixture) (group 1) or 100 times higher doses (100 × ADI mixture) (group 2), or were maintained on the basal diet throughout the experiment (group 3). Groups 4 and 5 were injected with saline and then fed on the ADI mixture diet and the 100 × ADI mixture diet, respectively. All animals were subjected to two-thirds partial hepatectomy at week 3 and sacrificed at week 8. Liver slices were fixed in ice-cold acetone, embedded in paraffin and then immunohistochemically stained for glutathione *S*-transferase placental form (GST-P) as previously reported [4,5,7]. Numbers and areas of GST-P positive hepatic cell foci larger than 0.2 mm in diameter and the total areas of liver sections examined were measured using a video image processor.

2.2. Experiment 2

Pesticides selected for the mixtures were 20 chemicals for which carcinogenicity has been reported or suspected (Table 2) and 40 chemicals of high volume production (Table 3). Tumor-modifying effects of pesticide mixtures were

Table 2
Information summary for the 20 pesticides for which carcinogenicity has been reported or suspected (Exp. 2)

Pesticides	ADI (mg/kg/day)	Mutagenicity (Ames test)	Carcinogenicity and target organs (species)
Insecticides (9)			
Acephate	0.03	+	Liver (mouse)
Dichlorvos	0.0033	+	Pancreas, leukemia (rat), stomach (mouse)
Dicofol	0.025	–	Liver (mouse)
Cypermethrin	0.05	?	Lung (mouse)
Permethrin	0.048	?	Liver, lung (mouse)
Phosmet	0.02	+	Liver (mouse)
Amitraz	0.0012	?	Suspected
Clofentezine	0.0086	?	Suspected
Propoxur	0.063	?	Suspected
Herbicides (5)			
2,4-D	0.3	–/+	Brain (rat)
Glyphosate	0.15	–	Kidney (mouse)
Trifluralin	0.0075	–	Multiple organs (rat, mouse)
Mefolachlor	0.097	?	Positive (rat)
Dichlobenil	0.004	?	Suspected
Fungicides (6)			
Captafol	0.05	–/+	Multiple organs (rat, mouse)
Propiconazole	0.018	?	Liver (mouse)
Fosetyl	0.88	?	Urinary bladder (rat)
Triadimefon	0.012	?	Suspected
Mancozeb	0.05	?	Suspected
Maneb	0.005	?	Suspected

?: not reported as far as we know.

Table 3
Information summary for the 40 pesticides of high volume production (Exp. 2)

Pesticides	ADI (mg/kg/day)	Ames test	Carcinogenicity[a]	Pesticides	ADI (mg/kg/day)	Ames test	Carcinogenicity[a]
Insecticides (17)				*Herbicides (10)*			
Acephate	0.03	+	Liver (mouse)	Glyphosate	0.15	–	Kidney (mouse)
Chlorobenzilate	0.02	?	Liver (mouse)	Metolachlor	0.097	?	Positive (rat)
Cypermethrin	0.05	?	Lung (mouse)	Sethoxydim	0.14	?	–
Permethrin	0.048	?	Liver, lung (mouse)	Thiobencarb	0.009	?	–
Clofentezine	0.0086	?	Suspected	Pendimethalin	0.043	?	–
Oxamyl	0.02	?	–	Metribuzin	0.0125	?	–
Chlorpyrifos	0.01	?	–	Quinclorac	0.029	?	?
Cyhalothrin	0.0085	?	–	Chlorpropham	0.1	?	?
Diflubenzuron	0.012	?	–	Bensulide	0.04	?	?
Pirimiphos-methyl	0.01	?	–	Bentazone	0.09	?	?
Bendicarb	0.004	?	–				
Malathion	0.02	?	–	*Fungicides (12)*			
Fenbutantin oxide	0.03	?	?	Propiconazole	0.018	?	Liver (mouse)
Cyfluthrin	0.02	?	?	Maneb	0.005	?	Suspected
Trichlorfon	0.01	?	?	Triadimefon	0.012	?	Suspected
Fenvalerate	0.02	?	?	Imazalil	0.025	?	–
Flucythrinate	0.0125	?	?	Zineb	0.005	?	–
				Vinclozolin	0.1215	?	–
Plant growth regulator (1)				Fenarimol	0.01	?	–
Mepiquat chloride	0.075	?	?	Flutolanil	0.08	?	–
				Metalaxyl	0.019	?	–
				Chinomethionat	0.006	?	?
				Pyrifenox	0.1	?	?
				Myclobutanil	0.012	?	?

[a] Target organs (species).
?: not reported as far as we know.

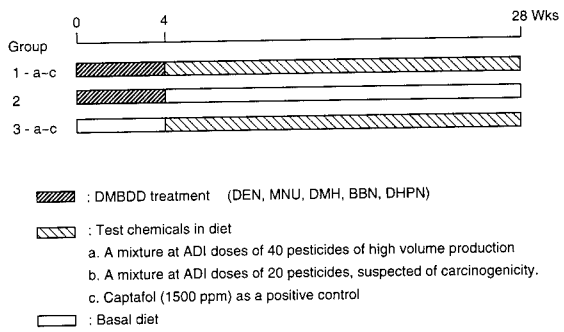


Fig. 2. Experimental protocol of the DMBDD model. Initiation treatment is a single i.p. injection of DEN at a dose of 100 mg/kg body weight at the start of the experiment, 4 i.p. injections of *N*-methyl-*N*-nitrosourea (MNU) at a dose of 20 mg/kg body weight on days 2, 5, 8, and 11, and 4 s.c. injections of 1,2-dimethylhydrazine (DMH) at a dose of 40 mg/kg body weight on days 14, 17, 20, and 23. The rats were additionally administered *N*-butyl-*N*-4-(hydroxybutyl)nitrosamine (BBN), 500 ppm in the drinking water during weeks 1 and 2, and 2,2′-dihydroxy-di-*n*-propylnitrosamine (DHPN), 1000 ppm in the drinking water during weeks 3 and 4. Pesticides were administered for 24 weeks.

investigated using the medium-term multi-organ bioassay (DMBDD model) [12,14]. As initiation, 5 known potent carcinogens were given in combination within the first 4 weeks as shown in Fig. 2. After this DMBDD treatment, groups of rats received one of the pesticide mixtures, captafol (1500 ppm in the diet) as a positive control [15], or the basal diet. Non-initiation controls were i.p. injected with saline and s.c. with corn oil and then given pesticide(s). At week 28 of the experiment, all surviving animals were killed and completely autopsied. Livers were analyzed as in Experiment 1. The small and large intestines, lungs, urinary bladders were inflated with 10% phosphate-buffered formalin, and other main organs and any macroscopic lesions were removed and fixed in formalin. The routinely prepared hematoxylin and eosin sections were examined for neoplastic and preneoplastic lesions.

2.3. Statistical analysis

Statistical analysis of differences between means was carried out using the Student's *t*-test or the Welchs *t*-test after application of the preliminary *F*-test for equal variance for each pair. For proportion data, the Fisher exact probability test was used.

3. Results

3.1. Experiment 1

No pesticide treatment-associated deaths occurred. Based on the data of food consumption values and average body weights, actual chemical intakes were found to be slightly lower than the estimated intakes. Body weights of rats initiated with DEN were similar in the 3 groups irrespective of the following pesticide treatment, and 6.3–7.1% lower than those of the non-initiated groups. Liver weights were also not influenced by the pesticide administration.

Data on the numbers and areas of GST-P positive foci per unit area of liver section with and without DEN-initiation are illustrated in Fig. 3. The number of GST-P positive foci in group 1 was $3.36 \pm 1.29/cm^2$ and the area of foci was 0.29 ± 0.15 mm^2/cm^2. The levels were essentially the same as those in the control group ($3.50 \pm 1.29/cm^2$ and 0.28 ± 0.13 mm^2/cm^2). However, the values obtained in the 100 × ADI mixture group ($4.51 \pm 1.64/cm^2$ and 0.44 ± 0.20 mm^2/cm^2) were both significantly higher than the control values. Without the DEN initiation, neither of the treatment schedules induced GST-P positive liver cell foci larger than 0.2 mm in diameter.

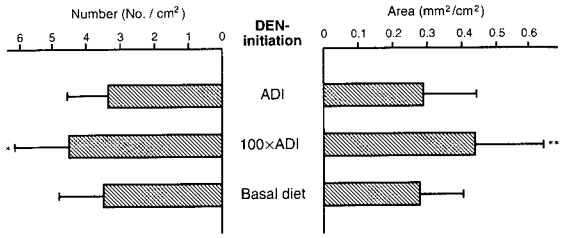


Fig. 3. Numbers and areas of GST-P positive liver cell foci in Experiment 1. No foci larger than 0.2 mm in diameter were observed in non-initiated groups. **,*Significantly different from basal diet group at $P < 0.01$, $P < 0.05$, respectively.

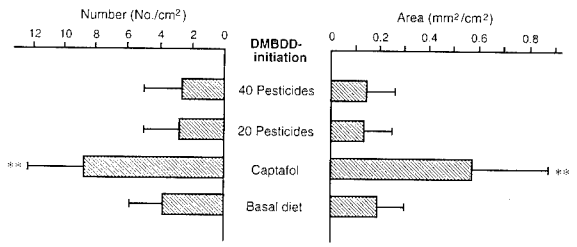


Fig. 4. Numbers and areas of GST-P positive liver cell foci in Experiment 2. No foci larger than 0.2 mm in diameter were observed in non-initiated groups. **Significantly different from basal diet group at $P < 0.01$.

3.2. Experiment 2

Only 1 rat of group 1-c died before the termination of the experiment. Final body weight was significantly decreased in group 1-c (captafol) and rather increased in group 1-a (20 pesticide mixture) compared to group 2. Relative liver and kidney weights were increased in the captafol group, but were not affected by treatment with the pesticide mixtures. Thus, no toxic effects were observed for pesticide mixtures in terms of survival rates and weight data. In the liver, GST-P positive foci development was increased by captafol but was not modulated by the mixtures (Fig. 4), however hyperplastic liver nodules were observed on hematoxylin and eosin stained sections (Table 4). In the other organs, captafol showed promotion effects in the thyroid, whereas the pesticide mixtures did not influence the neoplastic development in any organ (Table 4). No neoplastic and preneoplastic lesions were observed in non-initiated groups.

4. Discussion

In the liver model, the ADI mixture of organophosphorus pesticides exerted no effects on liver preneoplastic foci development initiated by DEN, although the 100 times higher dose mixture demonstrated lesion-promoting potential [13]. In the multi-organ model, the ADI mixtures of 20 or 40 pesticides demonstrated no tumor promoting potential in any organ or tissue. Captafol, on the other hand, exerted apparent tumor promoting effects in the liver and thyroid, although the dose level was not comparable to the mixtures. The protocols used for the present analysis is based on the 2-stage carcinogenesis hypothesis and has been developed in our laboratory over the last 15 years [12]. Quantitative analysis of GST-P positive foci larger than 0.2 mm in diameter, expressed in terms of number and area per unit area of liver section, has also been established. The multi-organ method has been developed to supplement the liver model and also demonstrated to be a useful method for rapid detection of carcinogens in a whole body level [12,14].

With a safety factor approach, acceptable exposure levels such as ADI are usually determined by dividing the no-observed-effect level (NOEL) from laboratory-based chronic toxicity tests by an appropriately chosen safety factor. In theory the safety factor reflects both the possibility of an increased sensitivity of humans relative to laboratory animals and the variation in susceptibility within the human population. The safety factor used for ADI by the Japanese Ministry of Health and Welfare and the FAO/WHO is usually 100, but the WHO expert committees have used figures ranging from 10 to 2000 [16]. Although there are a number of potential problems associated with the safety factor approach including the fact that the observation of no treatment-related effects may depend on the number of animals exposed and dose levels used, and biological justification for general use may be lacking [17], the present experimental results indicate that this procedure is indeed appropriate and acceptable for risk evaluation at present. Furthermore, the chance of exposure to so many pesticides (20 or 40 chemicals) in concert might be in practice very low [18].

The observed combination effects at 100 times higher doses in Experiment 1, however, suggest that several of the included pesticides are possibly carcinogenic in the liver. In fact, liver carcinogenicity has been reported for methidathion and malathion previously exerted enhancing effects in this system when given at a dose of 4000 ppm in the diet [7]. Even the mixture at

Table 4
Incidence of tumors (Exp. 2)

Organ and type of tumors	Group			
	40 pesticides	20 pesticides	Captafol	Control
No. of animals	20	20	19	20
Thymus				
Thymic lymphoma	0	0	0	1
Thyroid				
Follicular adenoma	2	6	9*	2
C-cell adenoma	1	1	0	0
Follicular carcinoma	0	0	0	1
Nasal cavity				
Papilloma	1	0	0	0
Odontoma	0	1	0	0
Adenocarcinoma	0	0	0	1
Lung				
Adenoma	4	5	3	5
Carcinoma	1	1	0	2
Oral cavity				
Odontoma	0	2	0	0
Esophagus				
Squamous cell carcinoma	0	0	1	0
Forestomach				
Squamous cell papilloma	3	8	2	4
Squamous cell carcinoma	0	1	1	0
Small intestines				
Adenoma	3	1	2	2
Adenocarcinoma	0	2	2	1
Large intestines				
Leiomyoma	0	1	0	0
Adenocarcinoma	2	2	6	4
Liver				
Hyperplastic nodule	1	0	1	1
Kidney				
Nephroblastoma	2	4	7	2
Transitional cell carcinoma	0	1	0	0
Urinary bladder				
Transitional cell papilloma	0	1	0	1
Prostate				
Leiomyosarcoma	0	1	0	0
Sarcoma, NOS	1	1	0	0
Seminal vesicle				
Adenoma	0	0	0	1
Skin/subcutis				
Squamous cell papilloma	0	1	0	0
Keratoacanthoma	0	0	0	1
Lipoma	0	1	0	0
Abdominal cavity				
Mesothelioma	0	0	1	0
Schwannoma	0	0	0	1
Peripheral nerve				
Schwannoma	0	0	0	2
Malignant schwannoma	0	1	0	0

* Significantly different from control group at $P < 0.05$.

ADI levels of 20 pesticides, for which carcinogenicity has been reported or suspected, exerted no tumor-modulating potential in the DMBDD model.

Possibly most human cancers may be caused by trace environmental factors. Therefore it is of increasing importance that combined effects of chemicals at relatively low doses be examined. It should be borne in mind, however, that since a clear dose-response relationship exists for complex mixtures as in each individual case, risk will decrease with decreasing dose so that measures taken to reduce chemical concentrations offer an effective approach to control. In conclusion, the present safety factor approach is appropriate for risk evaluation of environmental chemicals.

Acknowledgements

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Health risk assessment of chemical mixtures from a research perspective

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Abstract

Human exposures to chemicals in the environment and workplace typically involve chemical mixtures. One of the key risk assessment issues for mixtures is that of extrapolation from high to low dose. Observation of an interaction among chemicals in a mixture at high concentrations in animals does not necessarily mean that the same effect, in type or magnitude, will be significant in humans exposed to lower concentrations of the mixture. Physiologically based toxicokinetic (PBTK) models can be used to assist in the extrapolation from high to low dose. Mechanisms observed in animals such as competitive inhibition of xenobiotic metabolism (e.g., butadiene and styrene or benzene and toluene) can be incorporated into PBTK models. The models can then be used to predict the magnitude of the interactive effects at high and low exposure concentrations. The most relevant predictions can then be tested using selected experiments. A research strategy involving hypothesis generation through quantitative modeling and testing through laboratory-based experiments may be the most effective strategy for addressing the complex issue of human health risks from exposures to chemical mixtures.

Keywords: Chemical interactions; Risk assessment; Humans; Rats; Mice; Butadiene; Benzene; Styrene; Physiological models

1. Introduction

One of the critical human health issues of the 1990s and beyond is the toxicological interaction of chemicals from environmental, industrial, and dietary exposures. Issues of particular concern for chemical interactions include extrapolation from in vitro to in vivo situations, high to low doses, and animals to humans. In the case of in vitro to in vivo extrapolations, the dilemma is that many chemical interactions can be tested rapidly and with fewer animals by the use of in vitro systems. However, interactions found in vitro may not be relevant to in vivo situations. For example, chemicals can induce xenobiotic metabolizing enzymes in vitro, but an increased amount of metabolism will not always be observed in vivo. Chemical interactions may occur at high exposure concentrations where xenobiotic enzymes are saturated. However, these relevant mechanisms may not be significant at low exposure concentrations, where the xenobiotic metabolizing enzymes are not operating at their full capacity. And finally, as in all toxicology studies, we are faced with the need to extrapolate chemical interactions documented in animal

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studies to human exposure. Chemical interactions are most often demonstrated in animal studies, and yet questions arise as to whether specific mechanisms of action important for one laboratory animal are relevant to or operate in humans.

Physiologically based toxicokinetic (PBTK) models are valuable tools in assessing potential effects of exposure to chemical mixtures, particularly in the experimental design and hypothesis testing stage. Through the use of PBTK models, the potential for interactions among chemicals can be explored. Model predictions of the results of such interactions can then form the basis for hypotheses that can be tested experimentally. One advantage in using toxicokinetic models to predict the effects of chemical interactions is that many modeling experiments can be conducted. Model results can then be experimentally verified in the laboratory. In the end, this approach is much more efficient than a priori testing of numerous chemical mixtures, many of which may prove not to have any interactive effects.

PBTK models allow the articulation of a biologically accurate toxicokinetic description of the experimental animal model (Fig. 1). They incorporate correct flow and dose relationships. For example, multiple dose routes such as oral administration, inhalation, or injection can be incorporated into these models. These models also incorporate realistic tissue volumes, flow relationships, and solubility parameters for individual animal species and individual chemicals. Most important, the models incorporate metabolic pathways with measured kinetic parameters. Since one of the more important chemical interactions for a number of volatile organic chemicals is the alteration of metabolism, expression of metabolic pathways is critical for correctly predicting the possible nature of the interactive effect.

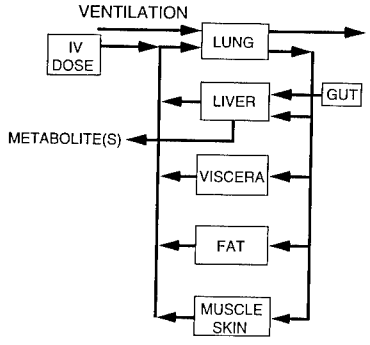


Fig. 1. A representative PBTK model.

Examples of interactions between butadiene and styrene demonstrate how model simulations lend themselves to the development of hypotheses that can be tested experimentally. Available experimental data in laboratory animals and humans on benzene-toluene interactions suggest that chemical interactions may be of toxicological significance.

2. Butadiene interactions

Styrene-butadiene mixtures are often encountered in the workplace where both of these monomers are used in the production of synthetic rubber. Unlike butadiene, styrene is highly soluble in tissues, especially lipid-rich ones. Oxidation of styrene to styrene oxide occurs by cytochrome P450 2E1 [1], the same P450 isozyme responsible for oxidation of butadiene to butadiene monoepoxide [2]. The biochemical interaction between these 2 chemicals was described in a physiological model by competitive inhibition of each chemical on the metabolism of the other [3]. The model used to describe interaction of styrene exposures on butadiene metabolism and vice versa used a single enzyme pathway for both chemicals and assumed strictly competitive inhibition. Thus, chemical and biochemical parameters for styrene and butadiene were known and incorporated into the model. Simulation of simultaneous exposure to butadiene and styrene predicted that the total amount of butadiene metabolized would be reduced but would not be proportional to the styrene exposure concentration (Fig. 2). In contrast, simulations predicted that butadiene was not an effective inhibitor of styrene metabolism due to the low solubility of butadiene in tissues (Fig. 2), an observation supported by experimen-

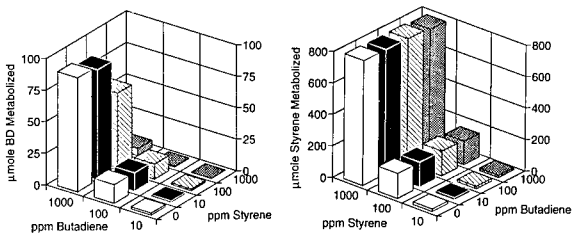


Fig. 2. Physiological model predictions for the micromoles of butadiene (left) or micromoles styrene (right) metabolized by rats exposed to various combinations of butadiene and styrene, assuming a simple competitive inhibition. Each bar represents model predictions of the amount of chemical metabolized both during and after 8-h simulated inhalation exposures to 1 of the 12 possible combinations of butadiene and styrene. For example, the effect of styrene on butadiene metabolism is simulated in the left-hand figure. As the inhaled concentration of styrene increases from 0 to 1000 ppm (right axis), the micromoles of butadiene metabolized due to coexposure to 1000 ppm butadiene (left vertical axis) decreases. For 1000 ppm butadiene, the largest amount of butadiene metabolized occurs with no exposure to styrene (0 ppm styrene). The least butadiene is metabolized when 1000 ppm styrene is present. Taken from Bond et al. [3].

tal studies [4]. The amount of styrene metabolized was only marginally affected by coexposure to butadiene.

In a study by Laib et al. [4], styrene decreased the rate of butadiene metabolism significantly for Sprague–Dawley rats coexposed to styrene and butadiene, but butadiene had no effect on the metabolism of styrene. Additionally, these researchers determined that the inhibition constant for styrene was much lower than its apparent Michaelis-Menten constant, suggesting that the interaction between butadiene and styrene might be more complex than the simple competitive inhibition description used in the model simulations shown here (Fig. 2).

From these simulations, one general point regarding chemical interactions should be noted. In extrapolations from high to low doses for the saturable enzyme systems that biotransform most toxic organic chemicals, it cannot be assumed that inhibition effects demonstrated at high exposure concentrations will be proportional to or even significant at lower exposure concentrations.

3. Benzene interactions

Benzene, an important industrial solvent, is also present in unleaded gasoline and cigarette smoke. Several investigators have demonstrated that a combination of benzene metabolites is necessary to duplicate the hematotoxic effect of benzene [5,6]. The dosimetry of benzene and its metabolites in the target tissue, bone marrow, depends upon the balance of activation processes such as enzymatic oxidation and deactivation processes such as conjugation and excretion. Enzymes implicated in the metabolic activation of benzene and its metabolites include the cytochrome P450 monooxygenases and myeloperoxidase. Other organic molecules that are also substrates for cytochrome P450 can inhibit the metabolism of benzene. The multiple metabolic pathways of benzene provide opportunities for modulation of benzene metabolism by competition with other organic chemicals for the available enzyme sites, by induction or inhibition of the oxidation or conjugating enzymes, or by direct competition between benzene and its metabolites.

Exposure to benzene causes genetic damage to bone marrow cells. Analysis of micronuclei in bone marrow cells provides information about recent bone marrow damage [7]. Micronuclei occur as a result of the exclusion of chromosomes from daughter nuclei during cell division. Gad El Karim et al. [8] investigated the genotoxicity of benzene in male and female mice treated with 2 oral doses of benzene or combinations of benzene and toluene. Benzene exposure produced elevated numbers of micronucleated bone marrow cells of both male and female mice compared to controls. When both benzene and toluene were coadministered, the number of micronuclei were reduced.

In an effort to correlate benzene toxicity and metabolism, other investigators administered radioactive benzene to mice with or without coexposure to toluene by s.c. injection [9]. Concentrations of benzene in various tissues (fat, liver, spleen, blood, or bone marrow) from mice given only benzene were very similar to those in which benzene was combined with toluene (Fig.

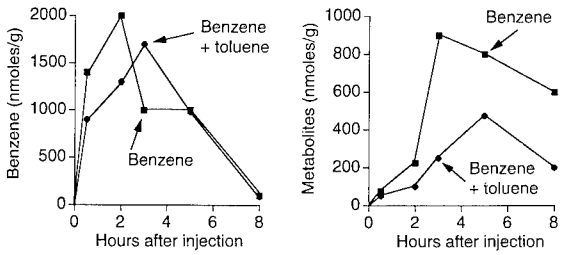


Fig. 3. Effect of coadministration of toluene on the accumulation of [^{3}H]benzene (left) or [^{3}H]benzene metabolites (right) in bone marrow of mice given s.c. injections of benzene (880 mg/kg) or benzene plus toluene (1720 mg/kg). Data taken from Andrews et al. [9].

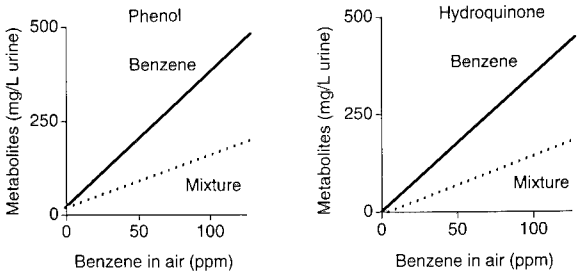


Fig. 4. Comparison of urinary excretion of 2 benzene metabolites, phenol (left) and hydroquinone (right) in workers exposed to benzene alone or mixtures of benzene and toluene. The regression lines describe the relationship between benzene exposure (TWA, ppm) and metabolite excretion (mg metabolite per l urine) for benzene-exposed (solid line) or toluene and benzene exposed (dashed line) groups. Data taken from Inoue et al. [10].

3). The similar benzene concentrations for benzene alone and benzene-toluene groups suggested that coadministration of toluene did not alter the disposition of benzene itself. In contrast, the concentration of benzene metabolites in tissues of mice given benzene alone were much higher than those found when benzene was coadministered with toluene. Coadministration of toluene did not delay the appearance of benzene metabolites in tissues but markedly reduced the concentration of metabolites found in each tissue for all time periods.

In both studies, toluene reduced the level of benzene metabolites in tissues and also reduced benzene-induced formation of micronuclei. Taken together, these observations suggest that the metabolism of benzene is closely related to its genotoxicity. Thus toluene may protect against benzene-induced genotoxicity by reducing the level of benzene metabolites in the bone marrow through suppression of benzene metabolism.

The previous studies demonstrated that coexposure to large bolus doses of benzene and toluene can reduce both the amount of benzene that is metabolized and the resulting benzene-induced toxicity in animals. The direct relevance of these experiments in predicting risk for humans is uncertain since human exposures are typically by inhalation at much lower exposure concentrations for longer durations. However, some evidence suggests that mutual metabolic suppression between benzene and toluene does occur in people exposed to concentrations of benzene and toluene in certain workplace environments.

For example, Inoue et al. [10] examined both the exposure concentration during a work shift and the benzene metabolite concentrations in the end-shift urine of male Chinese workers exposed to either benzene or toluene or a mixture of both chemicals. Additionally, these investigators looked at nonexposed male workers (control group). The relationship between the time-weighted average (TWA) benzene concentration and the concentration of the individual metabolites in urine was analyzed for each group (Fig. 4). The results indicated that urinary levels of the benzene metabolites phenol and hydroquinone were lower in the group exposed to both toluene and benzene compared to the group exposed to benzene alone. The investigators hypothesized that biotransformation of benzene to its hydroxylated metabolites in humans is suppressed by coexposure to toluene. These findings suggest that when workers are exposed to toluene in addition to benzene, the amounts of phenol and hydroquinone excreted in the urine will be lower than those expected from exposure to benzene only at similar concentrations.

4. Conclusion

The development of quantitative linkages between exposure and response based on biologically plausible mechanisms of action at exposure

levels that are likely to be encountered by people will significantly improve risk assessments for humans exposed to chemical mixtures. The use of PBTK models as tools to assist in predicting internal dose following exposure to chemical mixtures can prove to be valuable. Examples presented here for butadiene-styrene interactions demonstrate how these models can be used to predict potentially important interactions. Ultimately, experiments using whole animals can be used to verify model predictions of chemical interactions. Mechanistic research coupled with the development of dosimetry models can assist not only in the interpretation of effects of combined exposures but also in predictions of dose to target tissues.

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Risk assessment of chemical mixtures from a public health perspective[1]

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Abstract

Health risk assessment is the practice of evaluating the degree of danger associated with chemical exposure, whether the exposure is intentional (pharmacologic agents, pesticides) or unintentional (industrial/automobile by-products). Chemical exposure can either be to a single chemical or to complex mixtures such as industrial effluents, municipal wastes, jet fuels, gasoline, or mixtures of drinking water contaminants. The mixtures can be simple or complex; partially or completely characterized; and stable or varying in composition. Three different approaches are often used in health risk assessment of chemical mixtures (51 FR 33992-34054). These 3 approaches consist of (a) use of data on the specific mixture of concern; (b) use of data on a similar mixture; and (c) use of data on each component of the mixture. The individual component-based approach is by far the most often used because it allows the individual risks from each component to be combined, usually by dose or response additivity, to calculate an overall risk for the mixture. In addition, several innovative methods, such as the toxicity equivalency factor, relative potency, and even the use of indicator chemicals, are also employed. More recently, a binary weight-of-evidence approach has been proposed to evaluate potential interactions between the various components and to integrate them into the overall toxicity assessment of the mixture. Because no single approach is suitable for assessing the health risk associated with all the exposure scenarios associated with the various types of mixtures, the use of professional judgment is still imperative in conducting health risk assessments.

Keywords: Chemical mixtures; Toxicity assessment; Chemical interactions; Hazard index; Public health

1. Introduction

Health risk assessment is the practice of evaluating the degree of danger associated with chemical exposure. Assessing the risks associated with exposure to chemicals involves acquisition and interpretation of appropriate data; drawing and integration of conclusions from such data; and formulation of overall recommendations for the management of potential risks [1]. Chemical exposure could be intentional or unintentional and it could be to a single chemical or mixtures of chemicals. Ample examples of intentional exposure to chemical mixtures can be found in the pharmacology literature [2]. The potential of joint toxic action of chemicals has been long

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recognized by a diverse groups of scientists including physicians, public health practitioners, pharmacologists, toxicologists, pesticide scientists, health risk assessors, statisticians, mathematicians, epidemiologists and environmentalists. For this reason, several terms have been defined to explain the observations of joint toxic action such as those that are used to explain the various types of interactions that influence the toxicity; namely, synergism, potentiation, antagonism, inhibition, more than additive, additive and less than additive.

The concepts of additivity, synergism and antagonism have been successfully employed for a long time in the field of anesthesiology. For example, morphine in combination with other epidural anesthetics has been used effectively to provide improved overall pain relief following surgery. However, with dosing regimens of multiple anesthetics needed to reach levels necessary for total pain relief, the potential risk of side effects also increases. Thus, an ideal example of intentional exposure to chemical mixtures would be the multimodal pain therapy using 'balanced analgesia' [3]. This therapy is based on the hypothesis that a combination of (low-level) analgesics with different sites of action may improve overall post-operative pain relief with minimal or no side effects. This hypothesis was tested through an evaluation of the analgesic effects (pain relief) of adding low-dose clonidine to a very low-dose regimen of continuous epidural local anesthetics and morphine following hysterectomy [4]. Bupivacaine (5 mg/h) and morphine (0.1 mg/h) were given continuously with clonidine (18.75 μg/h) following a bolus dose of 75 μg. No significant differences were observed in pain scores at rest between the clonidine and placebo groups, but an enhanced analgesic effect was observed during cough and mobilization when clonidine was used.

An example of unintentional exposure to environmental chemicals would be the Gulf War syndrome that lacks a good case definition [5]. The exposure was to chemical/biologic agents, smoke and petroleum combustion products. This syndrome presented as a mixture of illnesses with substantial but unexplained manifestations that varied from person to person. Symptoms such as fatigue, abdominal pain, diarrhea, headache, memory loss, skin rashes, and hair loss were often reported. Exposure occurred through a combination of inhalation, oral and dermal routes. The exposure occurred under variable environmental conditions such as temperature, humidity, and high winds that blew sand. It was recognized that of the 700 000 regular military service personnel, several did not want to complain about health problems that were tolerable, because of possible adverse repercussions on their military careers. Also, about 150 000 personnel were given Anthrax vaccine while others were given a chemical warfare antidote known as pyridostigmine. Thus, this is a complex mixtures exposure that poses a formidable challenge for health risk assessors because of major data gaps and lack of information regarding characterization, duration, and route of exposure. The chemical mixtures encountered in the real world such as those found at hazardous waste sites are presented with information between (1) simple and completely characterized and (2) the highly complex and poorly characterized mixtures.

To assess the health effects of diverse mixtures encountered in the environment, 3 different approaches are often used in health risk assessment [6]. An appropriate method is used depending on the known toxic effects, the availability of toxicity data on the mixture or similar mixture, the known or anticipated interactions among components of mixture and the quality of exposure data (Table 1). The health risk assessors should utilize every plausible approach that can be applied, compare the results and decide to use the approach that best suits the exposure scenario. The results of such multiple analyses may be useful in describing the uncertainty in the risk assessment that is under consideration. In practice, the use of all 3 approaches may not be possible because of lack of data, time and other resources.

2. The mixture of concern approach

The first approach is the 'mixture of concern' approach. It is the most direct and simplest method and entails the fewest uncertainties.

Table 1
Risk assessment approaches used for chemical mixtures

	Mixture of concern	Similar mixture	HI
Efficiency and utility	+++	++	+
Frequency of use	++	+	+++
Toxicity of information	++	+	+++
Monitoring data availability	++	+	+++
Uncertainty[a]	+	++	+++

[a] Uncertainty in estimation of risk and influence of chemical interactions.
+++, most; ++, some; +, least.
HI, hazard index.

Hence, it can be called the preferred approach. This approach, however, is the one that can be least frequently applied because it requires that toxicity data be available on the specific mixture of concern to the risk assessment and that these data be adequate for deriving an integrated allowable level such as a minimal risk level (MRL) for the mixture. Such mixtures are few. Recently an MRL was derived for fuel oils, mixtures of aliphatic and aromatic petroleum hydrocarbons, that contain small amounts of nitrogen, sulfur, other elements and additives as well. While the exact chemical composition of such a mixture may vary to a small degree, it is reasonably consistent from sample to sample. The study used in the MRL derivation represented the actual controlled exposure to a well-defined mixture, and was considered to represent a 'best case' exposure scenario. The relevance of effects observed in laboratory animals during daily exposures may not always be extrapolatable to other human exposure scenarios, depending on the toxicokinetic properties of the components of the chemical mixture. For example, hepatotoxic effects were observed in mice exposed to jet fuel (JP-4) vapors at a concentration of 500 mg/m^3 for 24 h per day, 7 days a week for 90 days, but not in mice exposed for only 6 h per day, 5 days a week for 8 months at an air concentration of 5000 mg/m^3 or for 6 h per day, 5 days a week for 1 year at 1000 mg/m^3. Often it is not possible to derive a single risk assessment value that can be used directly in all risk assessments because some mixtures such as gasoline generally substantially vary in composition depending on the source of the crude oil or differences in the fractionation process.

3. The 'similar mixture' approach

This leads to the second approach used for the risk assessment of chemical mixtures. Although all the information may not be available on most complex mixtures, the toxicologic properties of some of them such as coke oven emissions, diesel exhaust, and wood stove emissions have been extensively investigated. Such information can be considered in the risk assessment and used if the mixture on which information is available is 'sufficiently similar' to the mixture of concern. For example, if the risk assessment is needed for gasoline contamination of groundwater and information is available on the chronic toxic effects of gasoline, it may be possible to use the available information to assess risks from the contaminated groundwater. However, there are no set criteria to help decide when a mixture is sufficiently similar. Hence, it is left to the judgment of the health assessor to balance the uncertainties inherent in the dissimilarities between the composition of 2 mixtures versus those in the other available assessment methods. One method that can be somewhat helpful in deciding the similarity is the relative potency method [7–9]. This method allows the use of information from short-term bioassays on the mixture of concern to conduct the risk assessment in the absence of more complete data thus helping to determine sufficient similarity. The relative potency method is based on the hypothesis that the 'relative potencies' of chemical mixtures are consistent across various bioassays used for toxicity testing of certain endpoints. When validated, this approach offers a method for using short-term assays of complex mixtures as a surrogate

for long-term in vivo assays. The approach first normalizes the results among each class of bioassays relative to some standard bioassay, thus the term 'relative potency' method. This method has only been specifically applied to complex mixtures that cause cancer and for which the dose response functions can be described by a simple linear dose-response model.

4. The hazard index approach

The first 2 approaches are used for those mixtures that have been experimentally tested as a whole. The goal of the third approach, the hazard index (HI) approach, is to approximate the toxicity of mixtures that have not been experimentally tested. This approach is based on the component toxicity of the chemical mixtures and attempts to construct an HI for the whole mixture and in fact, is the most often used approach. The HI approach attempts to combine exposure levels and toxicologic consequences of the exposure into a single value obtained by potency-weighted dose addition of each component of the mixture. It relies heavily on some form of addition of either doses or responses. The procedures involved in this combination are based on classical definitions developed in the toxicology of mixtures [10]. Similar joint action, as defined by Bliss [11] is the conceptual basis for the HI approach. This is also referred to as simple similar action. Similar joint action is 'non-interactive' (i.e., the chemicals in the mixture do not affect the toxicity of one another). This form of joint action also postulates that all the chemicals in the mixture act in the same way, by the same mechanism, and differ only in their potencies, i.e. the chemical components of a mixture behave as if they were dilutions or concentrations of each other, thus the true slopes of the dose-response curves for the individual chemicals are identical. In other words, the chemicals behave as concentrations or dilutions of one another. The general equation for the HI is:

$$\mathrm{HI} = \frac{E_1}{DL_1} + \frac{E_2}{DL_2} + \cdots + \frac{E_n}{DL_n} \qquad (1)$$

In Eq. 1, E_1 is the level of exposure to the first chemical in the mixture and DL_1 is some 'Defined Level,' often a regulatory goal, for exposure to the first chemical. Similarly, E_2 and DL_2 are the corresponding levels for chemical 2. This can be continued for any number of chemicals, signified by the n in Eq. 1. Each of the individual ratios (e.g., E_1/DL_1) is called the hazard quotient. The HI is the sum of the hazard quotients.

$$\mathrm{HI} = \sum_{i=1}^{n} \frac{E_i}{DL_i} \qquad (2)$$

In addition to its use in the HI, similar joint action also serves as the basis for the 'toxic equivalency factor' (TEF) method [6] used for chlorinated and brominated dioxins and dibenzofurans [12]. Like the relative potency method, the TEF method allows incorporation of information from short-term bioassays or other forms of toxicologic data that might not otherwise be directly useful. The TEF approach starts with the assumption that the components in the mixture are dose additive. All the available data on the components is used to judgmentally estimate the relative potencies in terms of a reference chemical. Potency estimates are used to convert the levels of various components in the mixture into equivalent doses of the reference chemical. This method is applied only to a toxicologic class of chemicals with similar mechanisms of action so as to allow judgmental correlation of the results from short-term assays to long-term toxicologic effects.

The HI approach is simple but limited with respect to the influence of chemical interactions on the overall toxicity, and estimation of adverse effects. Additivity assumptions can lead to substantial errors in risk estimates of mixtures of industrial, occupational and environmental chemicals, if synergistic and antagonistic interactions occur [13–18]. It has been found that each chemical component of a mixture has a unique potential to influence the toxicity of other mixture components. However, the magnitude or the capacity of this potential to interact is rarely known. Equally important is that the risk assessment values arrived at using the HI approach

should also express the available information on chemical interactions. There is very little guidance on how the interactions should be evaluated or incorporated in the overall risk assessment. The National Academy of Sciences has proposed the use of additional safety factors if synergistic interactions are of concern [19]. To provide further guidance on this issue, a binary weight-of-evidence (BINWOE) method can be used [20]. The BINWOE method yields a composite representation of all the toxicologic interaction evidence from animal bioassay data and human studies data; relevance of route, duration and sequence; and the significance of interactions. In terms of estimating risk, the HI values obtained using the HI approach should be interpreted carefully. For example, if chemical mixture 'X' yields an HI value of 4, it need not be interpreted as twice as toxic as mixture 'Y' that yields a value of 2. However, it can be said that mixture 'X' is more toxic than mixture 'Y'. Thus the HI approach can be used for priority setting of mixtures. Generally, as the value of the HI approaches unity, concern for the potential hazard of the mixture increases. In terms of estimating risk, it is important the estimates be realistic. Use of the defined value based on a critical effect for secondary effects assessment could overestimate risk [21]. To circumvent this problem, target-organ toxicity doses (TTDs) can be developed and employed [22].

5. Conclusion

Given the different types of mixtures for which health risk assessments are performed as well as the many factors that impact the overall toxicity of such mixtures no single approach is suitable to conduct every health risk assessment. An ideal risk assessment would be based on toxicologic data on the mixture of concern for which health effects have been well characterized thus requiring minimal extrapolation. Also, appropriate monitoring information either alone or in combination with modeling information is sufficient to accurately characterize human exposure to the mixture. But such characterizations of risk are rare. The risk assessment process must encompass all available toxicologic data and scientific evidence on the plausible toxicities of chemical mixtures. It is also imperative that research to develop appropriate methods continue with an emphasis on a 'systems' approach that studies multiple endpoints rather than specific endpoints. In the meantime, professional judgment that is gained by conducting such assessments has to be carefully used to ensure adequate public health protection. The chemical mixtures assessment process will benefit by utilizing a team approach wherein experimental scientists, model developers and health risk assessors participate toward the development of consensus.

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Regulatory and political perspectives in reproductive and developmental hazard assessment*

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Abstract

The World Health Organisation is attempting to harmonise the processes of risk assessment worldwide in the hope that assessments performed in one country will be acceptable to other countries. This would reduce not only duplication of work by scientists, but would also reduce to a minimum the need for animal studies. There are differences in the scientific approaches used in different countries to the process of hazard and risk assessment. For example, in the USA risk assessment is focusing on development of mathematical models to describe dose-response relationships to define, for example, a benchmark dose. In Europe much less use is made of such models. More dependence is placed on no-effect levels, and the use of safety factors is much more highly developed. Political considerations come into play when one looks at the use, or misuse, that may be made of such hazard and risk assessments. Once a chemical has been classified and placed on a 'list' of reprotoxic chemicals, the underlying criteria may be ignored and actions taken which are quite unjustified by the original scientific evidence.

Keywords: Regulatory toxicology; Risk assessment; Reproductive system

1. Introduction

The World Health Organisation (WHO) in collaboration with the OECD has started a programme to attempt to harmonise the risk assessment processes worldwide. The intention is not to attempt to standardise the processes but to develop an understanding of the methodologies used in the different countries and to develop confidence in the different methods used. Ultimately it may be possible to move towards the use of common methods. In an increasingly international environment with growing demands for reduction in animal experiments, it is important to avoid the duplication of studies merely because they do not conform to the guidelines of another country, or even worse, to the guidelines of another regulatory authority within one country. One of the aims of risk assessment must be to make the best use of available data, and to develop the most economic designs of animal study for this purpose.

One of the first tasks has been to attempt to harmonise the terminology used in the field of reproductive toxicology so that at least we all understand the meaning of the main terms used. There is increasing agreement between the USA and Europe that the term 'reproductive toxicity'

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should mean adverse effects on all the processes related to reproduction from sexual development and behaviour, through fertility and conception, and including development pre- and postnatally. For convenience this is frequently subdivided into 'sexual function and fertility' to refer to effects on the male and female sexual behaviour and gonads. This includes any effects from puberty to conception and development up to the stage of implantation. 'Developmental toxicity' has not been clearly defined but includes embryofetal development from the stage of implantation through parturition to full postnatal development up to the stage of puberty. These are the definitions which have been used in the EEC classification process for chemicals under the EEC Directive on Dangerous Chemicals.

'Risk Assessment' is a process which involves 3 phases: (1) hazard identification; (2) dose-response extrapolation; and (3) exposure assessment. This then allows an estimate to be made of the risk from a defined exposure to humans. What action is then taken following this assessment is called 'risk management' and is not the subject of this paper. Each one of the phases in the assessment is complex and open to many variations and provides the problems which have to be overcome before harmonisation can be achieved. Hazard identification usually involves the detection of reproductive toxic effects in animals. The precise characterisation of these effects is done in further, more detailed animal experiments and in humans. Dose-response extrapolation attempts to define the range of doses which cause, or which do not cause adverse effects. It also involves extrapolation within and between species and the use of modifying factors. Exposure assessment involves examination of the actual levels of a chemical to which humans are exposed and may need to take into account special features which may be of relevance, such as route of exposure.

2. Hazard identification

Hazard identification is normally done by means of animal experiments. Many different designs of study have been used to assess adverse effects on development and there has been a gradual change in recent years from studies designed solely to detect teratogenic effects to studies with a much wider range of endpoints. In particular there has been interest in adverse effects on postnatal development following exposure during pregnancy. This has necessitated modification of the older study designs and, in the testing of drugs for worldwide markets led to a huge increase in the work required. This has led to a major revision in the drug testing guidelines by the International Conference on Harmonisation and the new guidelines have been readily accepted, leading to a reduction in the numbers of animals used. Since regulatory requirements for most other types of chemicals, food additives, pesticides and industrial chemicals have been based on the drug-testing guidelines, it is likely that testing of these will change also. One basic study design used for these other chemicals which was not used for drugs, the multigeneration study, has also been modified extensively in recent years mainly by reducing the number of generations and litters tested from the original 3-generation, 2-litter protocol to a variety of usually lesser options.

It has to be remembered that regulatory guidelines are normally only for the detection of hazards, they are not designed for hazard characterisation. In order to assess whether effects detected in the animal studies have relevance for human exposure situations, it is necessary to carry out studies to attempt to characterise the effects, i.e. to know the dose-response relationships, the mechanisms by which the effects are induced, the relationship to other effects produced in the adult animals which may be relevant, and the species specificity.

3. Developmental studies

Problems which still have to be solved in achieving harmonisation of risk assessment include a number of issues relating to animal

testing. For example: how many species are necessary, and which are the best species? what can be done if the rat or rabbit are not suitable for some reason? what is the minimum number of animals which can be accepted? what should be the duration of dosing? which guidelines are acceptable if only a limited number of studies have been done? whether it is necessary to determine a no-effect level or whether a method like the benchmark dose calculation which does not require a no-effect level can be substituted instead.

4. Fertility studies

In females, the traditional methods of assessment by dosing for 2 or 3 cycles followed by mating trials is still the most common method. This may be supplemented when problems arise by assessment of cycles by use of vaginal smears. This gives a good indication of normal cyclical hormone activity.

In males however, there has been a change in attitude in recent years as to which methods are the most satisfactory for detecting adverse effects on the testis. In rats, there is normally a large excess of sperm production compared with the number required for fertility to be maintained. It is clear that there can be considerable testicular damage with reduction in testis weight, damage to the seminiferous tubules, reduction in sperm number and increase in non-motile or abnormal sperm, without any detectable change in fertility in the males as assessed by mating performance. This is clearly different from the situation in humans where relatively small reductions in sperm number may be associated with infertility. Increasing reliance has therefore been placed on histological and sperm quality assessments. This is demonstrated by the changes proposed in the ICH guidelines for drug evaluation and the new OECD guidelines for chemical assessment which have reduced the time of premating treatment of males from 10 to 4 weeks provided that no histopathology in the testis or epididymis has been observed. Actual mating studies are still required to assess sexual behaviour.

5. Assessment of the study outcomes

It is interesting to analyse why different scientists assessing the same study will frequently come to different conclusions on both the outcome of the study and on its significance for extrapolation to man. Some of the areas of disagreement are as follows:

(1) Should all differences from controls be regarded as adverse? Should treated groups be compared with concurrent controls or with historical control ranges, and if historical over how many studies/months/years? Can one compare within the same strain only within one laboratory, or between laboratories if the animals are from the same supplier, or from different suppliers (breeding colonies)?

(2) What importance should be accorded to results which are statistically significant but not biologically significant, or vice versa? What is the importance of a steep versus a shallow dose-response relationship? What if the treated groups differ from the controls but there is no dose-response relationship?

(3) Are some effects more important than others? Is this a function of their negative impact on health? Is persistence a factor – is reduced fetal weight important if the birth weight is not affected? Are effects like wavy or extra ribs important if they disappear by weaning? Is a reduction in mean birth weight as important as an increase in the proportion of low-birth-weight offspring? Is an increase in the proportion of common variants important, and is it more important if it has a very marked dose-response relationship? Would a marginally significant increase ($P < 0.05$) in gross malformations be important if the malformations were all of different types? Would it be more important if they were all identical? What difference would it make if the background rate was relatively high or extremely low?

All of these are difficult questions and are

given different weightings by different scientists. These considerations start to overlap with the final stages of risk assessment of extrapolation to humans and setting acceptable exposure levels or acceptable daily intakes by introducing different values for safety factors.

6. Extrapolation to humans

The most satisfactory situation is when one can define the mode of action of the reproductive toxicant in the animal model and then state whether such a mechanism would operate in humans. The differences in reproductive physiology between species are sufficiently large for there to be cases where a chemical affects fertility or development by acting on hormones or systems which do not operate in humans. In such a situation, the animal studies can be discounted, though the different effects in humans are still possible. If mechanisms similar to those responsible for the adverse effects in animals do exist in humans, then some confidence can be achieved by determining the no-effect levels for the underlying changes in human studies.

The more usual situation, however, is that the underlying mechanisms in animals are not known, and then one has to resort to the use of safety factors in arriving at the acceptable levels of exposure. This is the least scientific part of risk assessment, and is regarded by some as a 'numbers game' and by others as a scientific judgement. There are 2 apparently different approaches to this final stage:

(1) Determine an acceptable daily intake (ADI) for food chemicals, or equivalent such as the 'tolerable daily (or weekly, monthly) intake' for environmental toxicants, or in the USA a 'reference dose' (Rfd, which is essentially the same as the ADI). All of these are estimates of the amount of chemical which if consumed regularly over a lifetime would not be expected to produce any harmful effects.

This is calculated by taking the no-effect level (or some variant of it such as the no-adverse-effect level, or the benchmark dose) and dividing this by a safety factor or uncertainty factor.

(2) The other approach is to calculate a 'margin of safety' (or in the USA the 'margin of exposure' which is exactly the same). This is calculated by dividing the no-effect level by the exposure level.

From the point of view of international harmonisation the latter approach is more attractive since the calculation is based on factual data and no agreement is needed on the size of safety factors to be used. In practice, however, both approaches are the same since the margin of safety has to be assessed from the point of view of adequacy, i.e. is it high enough? This could be left to individual countries to work out. In an economic community like the EU, however, in order to guarantee free trade between countries, the safety margin would still have to be agreed internationally in, for example, the case of food additives.

7. Safety factors

This is another area of disagreement. Historically, the figure of 100 has been used in the case of food additives, but for pragmatic reasons smaller safety factors have been used for occupational exposure limits. It is generally accepted that the value of 100 is the multiple of 10 for intraspecies differences, and 10 for interspecies differences. This allows a scientific approach to be used for moderation of these factors to allow a safety factor different from 100 to be used. Primarily, consideration of pharmacokinetic and pharmacodynamic (or toxicokinetic and toxicodynamic) factors can be used in this process. For example, for substances which exert their toxic action without metabolism playing a role, e.g. metals, then a factor less than 10 might be used for within species differences. Similarly where the mechanism of toxic action is identical in all species studied, including man, then a factor less than 10 might be used for between species differences. The 2 numbers which have been used in recent studies have been 10 and 3 (in some USA papers 3.16, being the square root of 10 is a preferred number). This gives a series of numbers like 10, 30, 100, 300, etc. for use as

safety factors. In addition to the safety factors a number of other factors have been added in to the equation. Factors are allocated to take account of deficiencies in the studies, e.g. if the quality of the study is poor, if a low-observed-effect level (LOEL) has to be used rather than a no-effect level, and so on. Other factors may also be included in the calculation as modifying factors to take account of the types of toxic effect involved. For example, if the chemical produces very marked teratogenic effects in a high proportion of animals treated then an extra factor may be included. Similarly, if potentially irreversible effects are produced in the testis, such as loss of spermatogonia, this would give rise to use of a higher factor than if only late sperm stages were affected.

In the past it has been the practice in Europe to use some kind of overall judgement in arriving at the final safety factor to be used in deriving the acceptable levels of exposure, usually around 100 or occasionally 200–1000. Where the exposed population is small, a smaller factor may be used than when the exposed population will be very large. More recently, however, attempts have been made to categorise the factors allocated for each of the steps including the uncertainty and modifying factors, and values less than 100 are being proposed where the quantity and quality of the evidence seem to justify such figures. Perhaps the increasing use of such explicit calculations will improve the chances of international harmonisation in the risk assessment process.

8. Dangers of misuse of hazard-based lists

Under recent European legislation there is a requirement under the Dangerous Substances Directive to prepare a list of chemicals that are defined as 'toxic to reproduction' [1], and when classified there is a requirement to use specified labelling on the products. This list is specifically prepared using only 'hazard' as the criterion and with no regard to 'risk'. The definition of how this is assessed states that toxic effects on reproduction must be specific and not secondary to other toxic effects which may be observed at high doses. The document then elaborates briefly on how this may be attempted. The intention of such a list is to warn workers and others exposed to the chemicals of possible risks. It was implicitly assumed that under actual exposure conditions, risk assessment would be carried out so that suitable precautions could be put in place.

Unfortunately, such lists are subject to abuse by well-intentioned people who do not have the scientific background to understand how they were prepared, and their limitations. Two examples of such dangers can be cited. A group within the European Commission who were preparing a directive on the safety of pregnant women at work proposed that a blanket ban should be established on women working with any listed substance. This was changed at an early drafting stage. A second and more serious example is from another draft directive on 'Restriction on Marketing and Use'. It stated that any listed substance in Categories 1 or 2 should not be permitted for sale to the general public. This was based on a misunderstanding of the system. When the Commission was questioned, it responded, "well it is a good idea not to sell reproductive toxic substances". The concepts of exposure, safety margins and thresholds were clearly unknown to them. Anyone familiar with the Delaney Amendment in the USA would recognise the folly of such a rule for all consumer products, yet this directive is now becoming part of European law with only slight change.

It is very important that we think carefully before we become involved in preparation of lists of hazardous chemicals that might be misused. Remember the old Scottish saying "The best laid schemes of mice and men gang aft agley" (... go often wrong).

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Toxicology Letters 82/83 (1995) 539–547

Toxicology Letters

Physiologically based pharmacokinetic models applicable to organogenesis: extrapolation between species and potential use in prenatal toxicity risk assessments

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Abstract

A physiologically based pharmacokinetic (PBPK) model describing the disposition of 2-methoxyacetic acid (2-MAA; the proximate toxicant derived from oxidation of the ethylene glycol ether, 2-methoxyethanol) was developed in pregnant rodents. This model was validated with pharmacokinetic (PK) data from dams and embryos during major organogenesis. A physiological model of human pregnancy was then combined with the PBPK model and linked to an empirical 2-MAA PK model with 2 maternal compartments and a single or multiple conceptus compartment, depending on the developmental stage. This approach is intended to allow more realistic human pregnancy risk assessments by refining the reference dose calculations via uncertainty factors. It will be possible to eliminate an uncertainty factor of 10 for interspecies extrapolations in the 2-methoxyethanol risk assessment if the PBPK model described here is used.

Keywords: Physiologically based pharmacokinetics; Rodent gestation; Human pregnancy extrapolations; Pregnancy risk; Ethylene glycol ether teratogenicity; 2-Methoxyacetic acid; 2-Methoxyethanol developmental toxicity

1. Introduction and issues

There is presently no generally accepted quantitative model for extrapolating the risk of adverse prenatal effects elicited by chemicals from either high to low doses or across species. Therefore the risk assessment is more qualitative and, as conducted by the United States (US) Environmental Protection Agency (EPA), follows the guidelines issued by that agency for estimating the risks of suspect developmental and reproductive toxicants. This approach was used by the Occupational Safety and Health Administration (OSHA) of the US in the case of certain ethylene glycol ethers to determine those levels of occupational exposure below which significant risk of adverse health outcomes are unlikely [1].

Development of mechanism-based, quantitative dosimetry models that accurately predict chemical disposition in laboratory animals is an important component of the effort to improve exposure-response assessment. In physiologically based pharmacokinetic (PBPK) models, the compartments represent the physical structure of the organism [2]. To describe pregnancy, O'Flaherty et al. [3] developed a PBPK model of rodent

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gestation that incorporates growth and aging of the dam and conceptus. In that model, pregnancy-induced changes in maternal tissue volumes and blood flows, and growth of the embryo/fetus are depicted from conception to parturition. Development of a PBPK model which describes chemical disposition in the pregnant rodent is a necessary precursor for similar efforts with other species, including humans.

A PBPK model has now been developed to describe 2-methoxyacetic acid (2-MAA; the primary metabolite and the proximate toxicant of 2-methoxyethanol (2-ME)) dosimetry at a single day in mouse development [4]. The pregnancy model [3] served as the template onto which a description of 2-ME/2-MAA pharmacokinetics (PK) in the pregnant CD-1 mouse was superimposed. The resulting PBPK model successfully simulated the PK behavior of 2-ME and 2-MAA in maternal plasma, embryo and extraembryonic/amniotic fluid (EAF) on gestation day (GD) 11 [4]. By expansion of the PK database for other gestation times, the model's ability to simulate 2-MAA disposition at different developmental stages has now been validated [5].

The overview presented here focuses on the disposition and its mathematical model simulations of a chemical teratogen during early, middle, and late mouse organogenesis (GD 8, 11 and 13). An expanded 2-MAA PK database was generated at several stages of embryonic development. The PBPK model gave rise to several hypotheses as to how the biological data could be explained. Models that described active transport and reversible binding provided the best simulations of chemical disposition at both GD 11 and 13. The 'Active Transport Model' established a pumping mechanism for the chemical's disposition and resulted in 2-MAA flow asymmetries consistent with the PK data. The 'Reversible Binding Model' described binding in the embryo and EAF compartments characterized by a binding maximum and a dissociation constant. The PK of 2-MAA on GD 11 and 13 were accurately simulated by these alternative models, suggesting that active transport and/or reversible binding may play an important role in the kinetic behavior of 2-MAA at those times in mouse gestation. This insight has led to experimental evaluation of one of the plausible mechanisms (active transport of 2-MAA) that is now being tested experimentally.

All technical details of PK and 2-MAA partition coefficients have been previously described [4]. 2-MAA dosimetry data were obtained in GD 8 maternal plasma and conceptuses (consisting of combined embryo, EAF, yolk sac and decidual swelling) following an i.v. administration of 2-ME [6]. Data for GD 11 [4] and 13 following bolus oral and i.v. doses, respectively, were obtained in maternal plasma, embryo, and EAF. All aspects of the PBPK model are described in detail in a recent publication of the expanded mouse gestation model [5].

2. Results and discussion

For model discrimination, each simulation trial incorporated appropriate information regarding maternal and embryo age, route of exposure, and tissue partitioning. The following versions were assessed for their ability to simulate the biological data.

2.1. Flow-limited, single compartment model

On GD 8, this model (Fig. 1A) produced reasonably accurate simulations of both maternal plasma and conceptus data (Fig. 2A and B).

2.2. Original hypothesis: flow-limited placental transfer

When the original version of the model [4] for GD 11 was evaluated using the expanded database, it failed to accurately simulate 2-MAA kinetics on GD 13.

2.3. Flow-limited, multi-compartment model

On GD 11 and 13 this model underpredicted all embryo data for 2-MAA by at least 40% (data not shown). Therefore, other explanations were sought to explain the biological data.

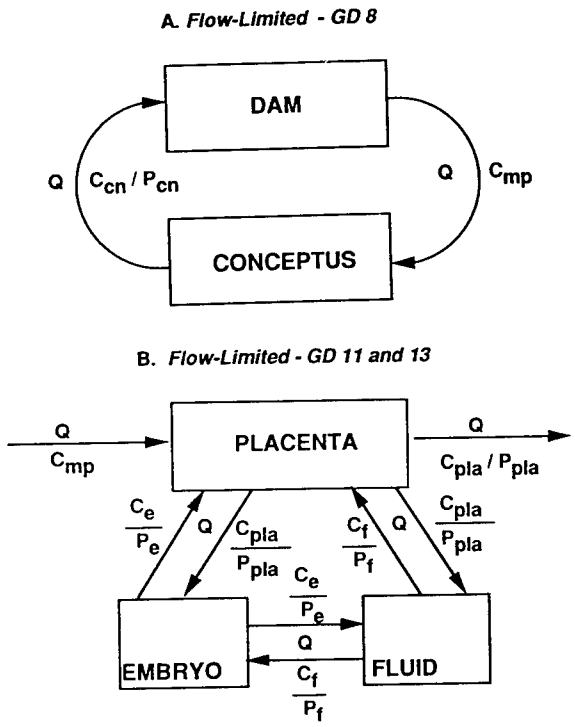


Fig. 1. Model variants for mouse gestation PBPK model. (A) Flow-limited – GD 8 compartmental model for 2-MAA. The dam is represented by a non-physiological compartment characterized by a volume of distribution for 2-MAA. The blood flow rate Q to the conceptus is the placental blood flow rate determined by the physiological model for pregnancy. The conceptus compartment, representing the lumped decidua, placenta, embryo, and extraembryonic fluid, has a volume defined by the physiological pregnancy model. C_{mp}, concentration (mmol/l) of 2-MAA in the dam; C_{cn}, concentration (mmol/l) of 2-MAA in the conceptus; P_{cn}, conceptus:blood partition coefficient. (B) Flow-limited – GD 11 and 13 model for 2-MAA. Volumes of placenta, embryo, and fluid are determined by the physiological pregnancy model. Flows between compartments (Q) are the same as blood flow to placenta (Q). C_e, C_f and C_{pla}, concentrations (mmol/l) of 2-MAA in embryo, extraembryonic fluid and placenta, respectively. P_e, P_f and P_{pla}, tissue:blood partition coefficients for embryo, extraembryonic fluid and placenta, respectively.

2.4. Alternative model hypotheses

2.4.1. pH trapping, multi-compartment model. Both on GD 11 and 13, this model (not shown here; for details see [5]) failed outright; the simulations predicted negligible chemical accumulation in the embryo and EAF under physiological pH conditions.

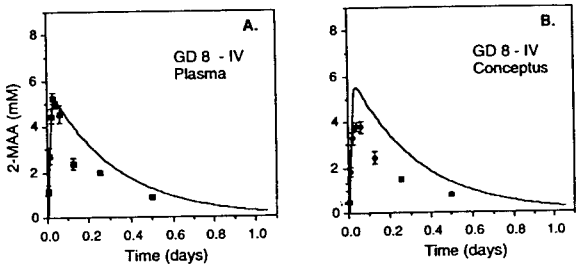


Fig. 2. Single compartment model for the GD 8 conceptus. 'Flow-Limited Model' simulations (solid lines) of 2-MAA PK data points (±S.E.M.) for the maternal plasma (A) and conceptus (B) following IV injection of 2-ME.

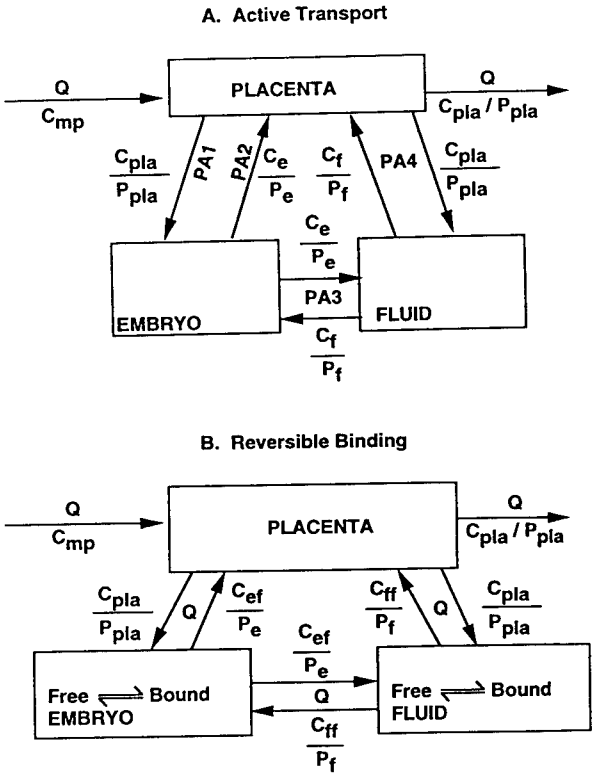


Fig. 3. Alternative model hypotheses. (A) Compartmental model diagram depicting active transport. Blood flow between compartments (Q) is replaced for the embryo and extraembryonic fluid by compartment specific flow parameters, PA1, PA2 and PA4. In the active transport version, these flow parameters (PA values) established asymmetrical 2-MAA movement. (B) Compartmental model diagram depicting reversible binding. In the reversible binding description, chemical transport was flow-limited (Q = Q), but a carrier molecule sequestered 2-MAA such that it existed in 'free' and 'bound' forms. C_{ef} and C_{ff}, concentrations of free 2-MAA in embryo and extraembryonic fluid.

2.4.2. Active transport, multi-compartment model. On either GD 11 or 13, this version (Fig. 3A) successfully simulated 2-MAA dosimetry in all compartments (Fig. 4A–F). Flow parameters (PA values; l/day) for both days were PA1 = 1.15, PA2 = 0.63, PA3 = 0.70, and PA4 = 0.30.

2.4.3. Reversible binding, multi-compartment model.
In this modification (Fig. 3B), reversible 2-MAA binding in the embryo and fluid was modeled on GD 11 and 13 (Fig. 5A–F) and provided reasonable simulations of GD 11 and 13 data. The binding capacity on both GD 11 and 13 was 0.0008–0.008 mmol. The dissociation constant was 0.0008 mmol/l on GD 11 and 0.003 mmol/l on GD 13. The model describing reversible binding generated predictions that were very similar to those obtained with the active transport model (compare in particular Fig. 4D–F with Fig. 5D–F).

Extrapolations of the mouse gestation PBPK model to pregnant rats was accomplished by the incorporation of data regarding the physiology of rat pregnancy. The model simulations were validated with biological data of maternal and embryo PK of 2-MAA collected on GD 13 and 15. Rat digits are maximally susceptible to 2-ME on GD 13 [7]. During the rat simulations it became apparent that the fit of the biological data to the model predictions could be significantly improved by dividing the maternal compartment into 2 compartments. One consisted of the richly perfused tissues, the other the poorly perfused tissues of the maternal body (Fig. 6). When the mouse simulations were repeated with this maternal body 2-compartment division, the fit of the simulations was noticeably improved

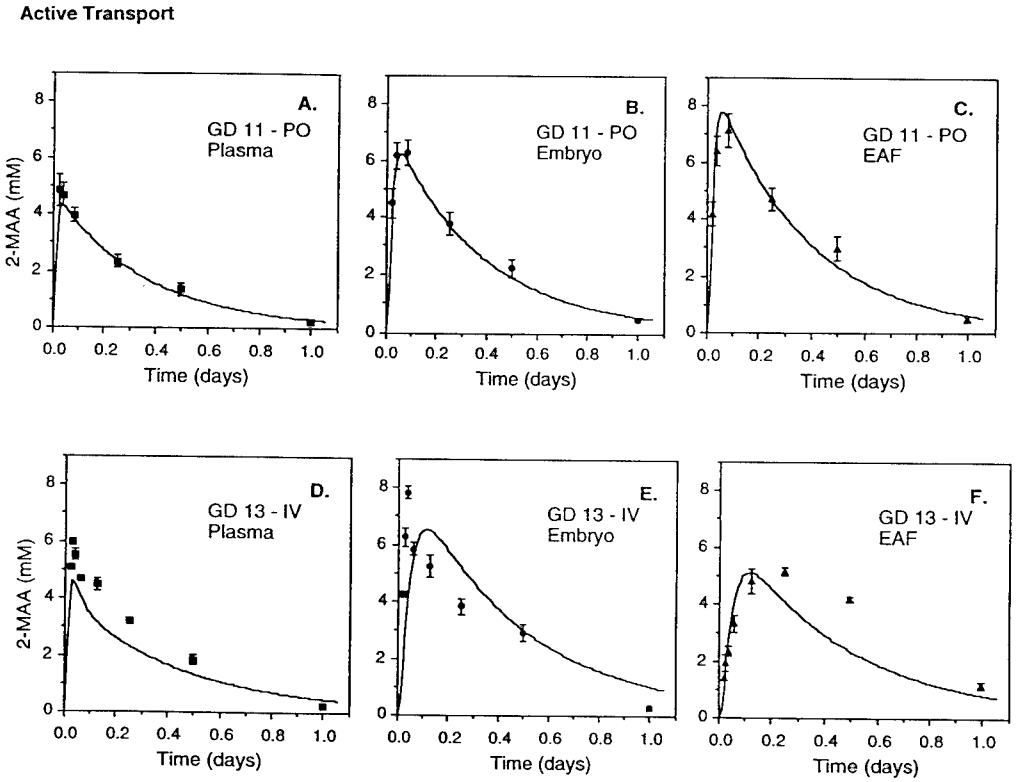


Fig. 4. Evaluation of alternative multi-compartment models. 'Active Transport Model' simulations (solid lines) of 2-MAA PK data points (±S.E.M.) in maternal plasma, embryo, and EAF on GD 11 (A–C) when 2-ME was administered by gavage (PO) and on GD 13 (D–F) when 2-ME was injected IV.

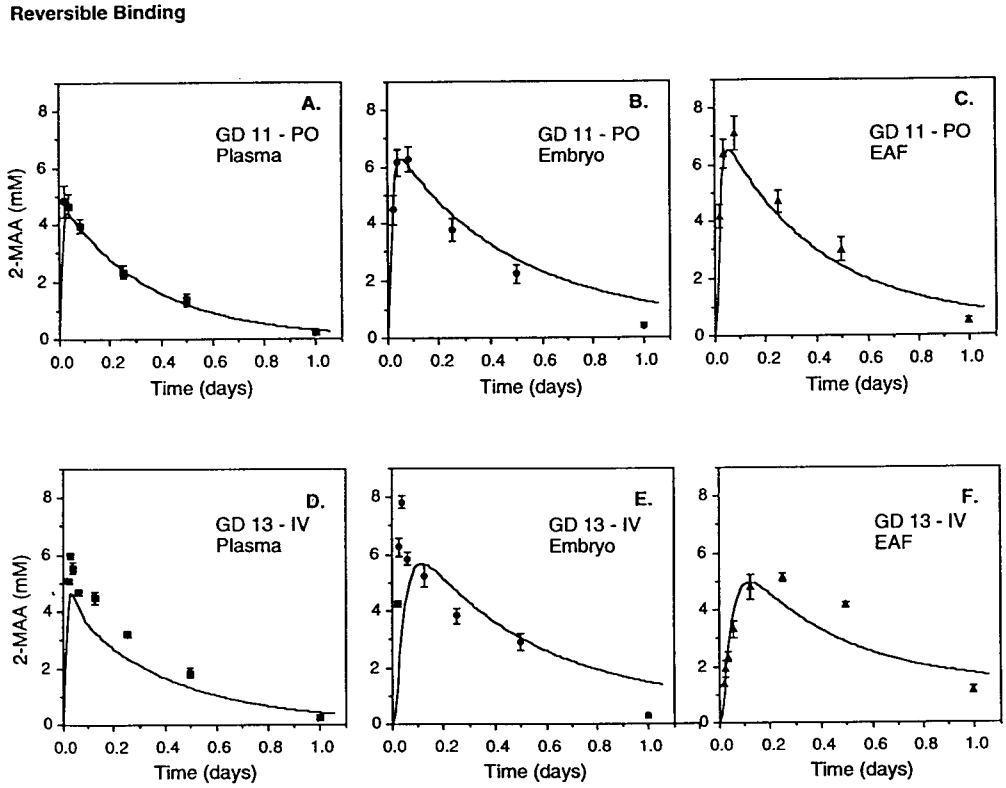


Fig. 5. Evaluation of alternative multi-compartment models. 'Reversible Binding Model' simulations (solid lines) of 2-MAA PK data points (±S.E.M.) in maternal plasma, embryo, and EAF on GD 11 (A–C) when 2-ME was administered by gavage (PO) and on GD 13 (D–F) when 2-ME was injected IV.

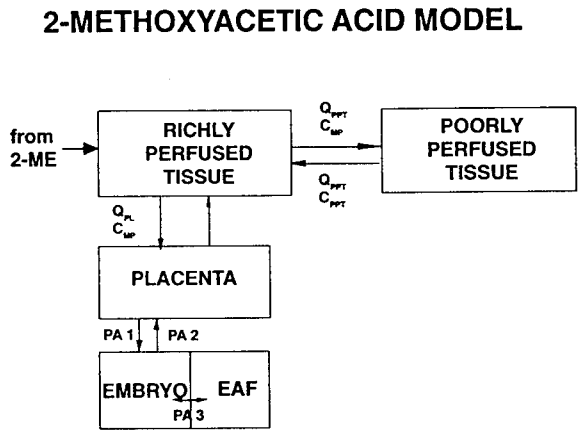


Fig. 6. Revised 2-compartment maternal body configuration. Division into separate poorly and richly perfused tissue components caused significant improvement of the agreement between model simulations and the biological data of 2-MAA disposition in mice and rats.

(Fig. 7A–C). The simulations of 2-MAA disposition in pregnant rats and their embryos turned from acceptable to very satisfactory (Fig. 7D–F) once the maternal 2-compartment modification had been introduced into the mathematical equations (Blumenthal et al., in preparation).

The next step in model development was extrapolation to human pregnancy. A physiological model of human pregnancy (kindly provided by Drs. Michelle Andriot and Ellen O'Flaherty, University of Cincinnati) was coupled to the 2-MAA disposition model that had been developed and validated in the 2 rodent species. The following biological data were available for inclusion into the human model: tissue growth characteristics during human pregnancy; blood flow to various tissues including the embryo during pregnancy; rates of oxidation of 2-ME to 2-MAA from in vitro experiments; and the 2-

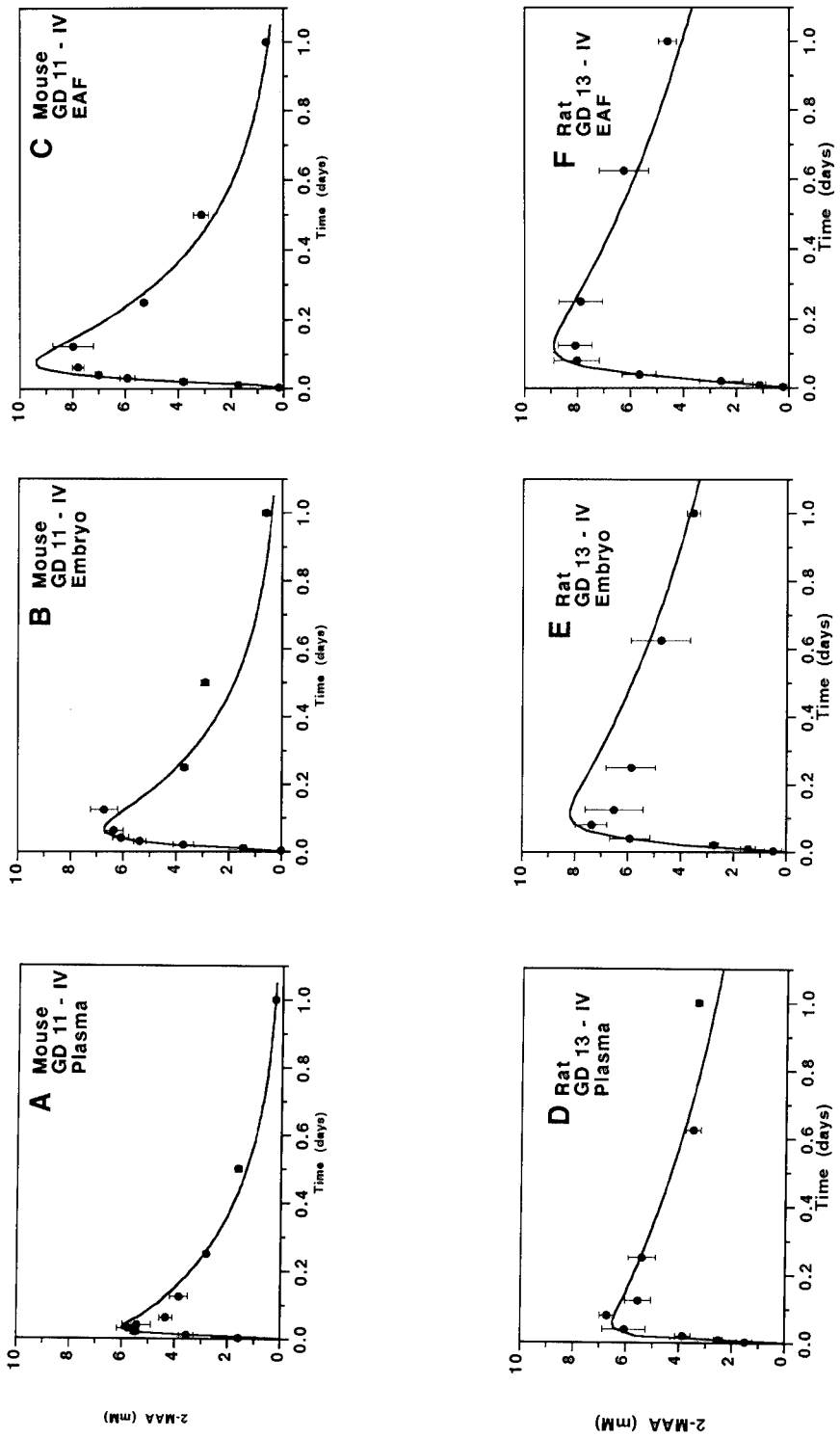


Fig. 7. Model simulations of GD 11 mouse PK data of 2-MAA (A–C) and GD 13 rat data (D–F). The solid lines are the simulations while the symbols represent the biologicall data points.

MAA elimination rate constant of ~77 h determined in humans [8]. For modeling purposes, the assumption was made that a likely route of entry of 2-ME into the human body would be by inhalation.

First, a 6-h in vivo inhalation exposure study at the present ACGIH-recommended threshold-limit value of 5 ppm was conducted in pregnant mice on GD 11. This regimen caused end-of-exposure concentrations of 2-MAA in maternal plasma approaching 50 μM and embryo as well as EAF levels exceeding the value in maternal plasma as is characteristic for mice on GD 11 (Fig. 8). Simulated inhalation exposures at 5 ppm, 8 h/day, 5 days/week over 3 consecutive weeks with typical weekend breaks in pregnant mice (Fig. 9A), rats (Fig. 9B) and pregnant women (Fig. 9C) revealed the impact of the profound differences in 2-MAA elimination half-lives on the accumulation of 2-MAA in maternal plasma. That T 1/2 value is ~6 h in mice [6] and ~24 h in rats (Blumenthal et al., in preparation). We have described that 2-MAA embryo dosimetry-teratogenicity relationships vary at different stages of mouse gestation. On GD 8, maximal concentrations of 2-MAA in dam and embryo (Fig. 10A) appear to determine the

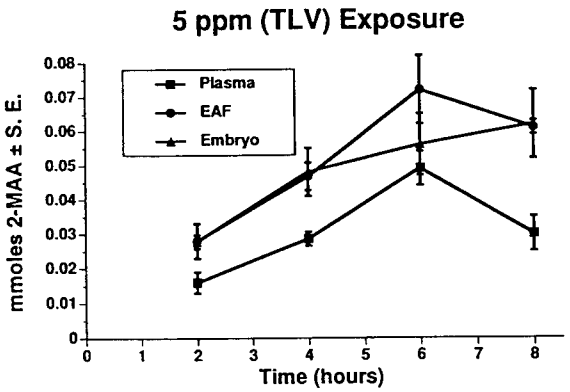


Fig. 8. 2-ME inhalation exposure of mice on GD 11. The animals inhaled 5 ppm 2-ME for 6 h. Mice were removed from the inhalation chamber for determination of 2-MAA levels in plasma, embryo and extraembryonic fluids at 2, 4, and 6 h into the exposure and 2 h (8-h time point in the figure) after termination. The ordinate shows the 2-MAA levels in target tissues expressed in mmol/l plasma and EAF or mmol/kg embryo.

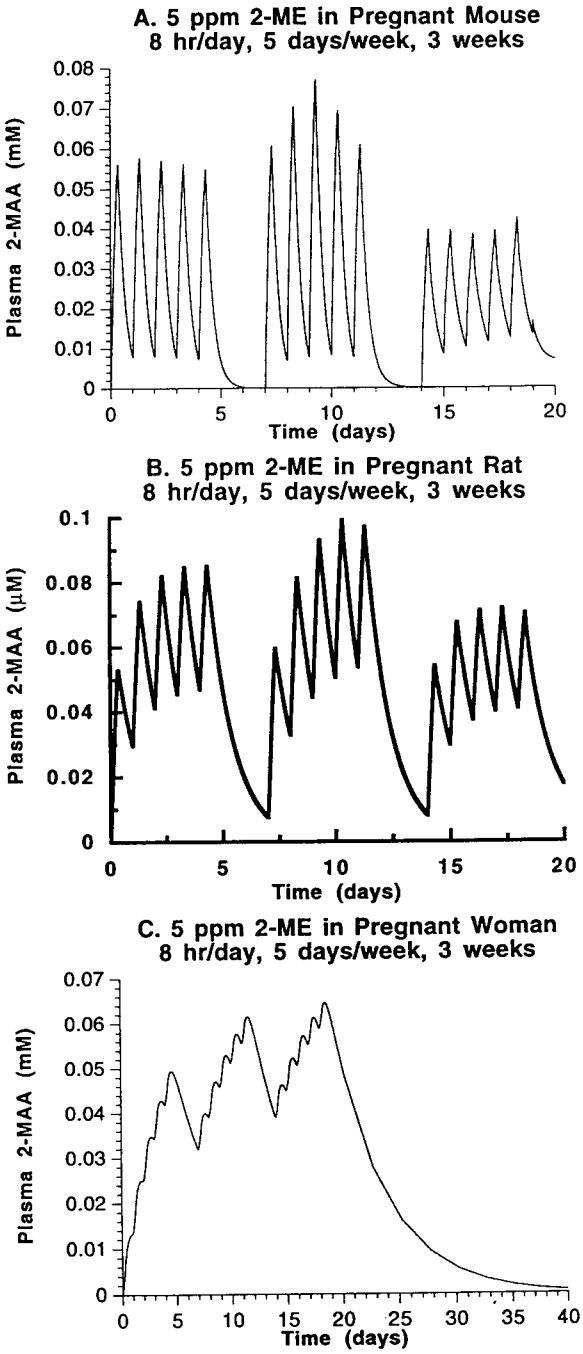


Fig. 9. Simulations of the PBPK gestation model in pregnant mice, rats, and humans. The underlying assumption is inhalation exposure to 2-ME at 5 ppm for 8 h/day on 5 consecutive days/week for 3 weeks. Each panel (A–C) illustrates the impact of the species-specific elimination half-life of 2-MAA from the maternal plasma, which is an excellent surrogate of embryo exposure.

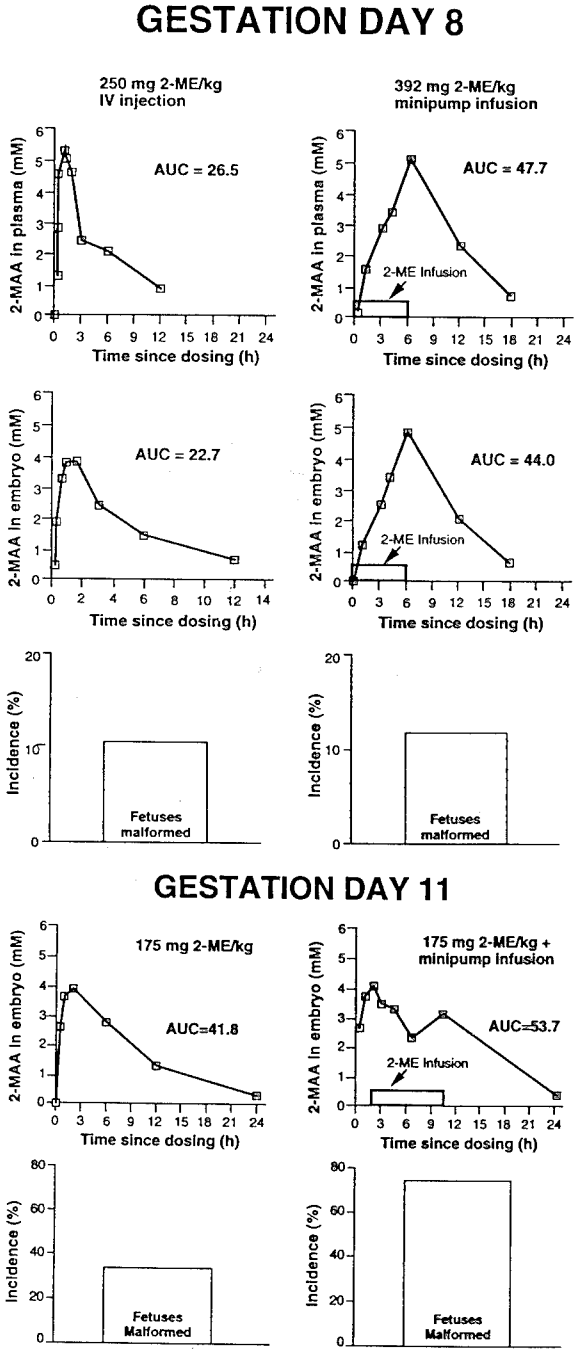


Fig. 10. Concentration vs. time profiles of 2-MAA. (A) GD 8 PK of 2-MAA in maternal plasma (upper panels) and embryos (center panels). These values are related to the malformation incidence (exencephaly) depicted in the lower panels. (B) GD 11; upper panels show the PK (C_{max} and AUC values) in pooled mouse embryos; lower panels depict digit malformation incidence.

incidence of exencephaly [9]. In contrast, on GD 11 total 2-MAA exposure concentration over time (AUC) seems to correlate much better with digit malformation incidence than maximal concentration of 2-MAA does (Fig. 10B). In conjunction with long elimination half-life of a developmental toxicant, as is the case with 2-MAA in humans, this chemical specific property will lead to accumulation of the toxicant upon daily repeated or continuous exposure to 2-ME. This kind of pattern could apply in the case of occupational exposure to 2-ME. Thus exposure levels that appear to be low compared to those applied in experimental animals may potentially cause developmental toxicity as a result of accumulation. At the end of the fifth day of exposure, human plasma concentrations reached ~50 μM and were thus comparable to those achieved in the simulated exposures of rats and mice after 8 h (compare Fig. 9A–C). Our large database collected in pregnant mice indicates that teratogenic levels are reached when the maternal plasma levels of 2-MAA are >1 mM [6]. Simulations of repeated inhalation exposure in a pregnant woman at the now proposed, much reduced U.S. OSHA permissible exposure level of 0.1 ppm, down from the previous 25 ppm OSHA standard [1] caused accumulation of 2-MAA to levels of the order of 1 μM (Fig. 11). This concentration was well below that which causes developmental toxicity in laboratory animals.

The observations summarized here show that the PBPK gestation model allows dramatic quantitative improvement of the database that could be used in risk assessment. The physiology of human pregnancy is built into the model, and it will therefore be possible to reduce the uncertainty factor to 3 to account for differences in pharmacodynamics when conducting interspecies extrapolations [10]. Since the mechanisms of the toxic action of 2-MAA are still unknown, this PBPK model remains empirical. The data shown here offer encouragement that the PBPK gestation model approach, which used 2-MAA as a prototypical developmental toxicant that is relevant to human occupational 2-ME exposure, has the potential to facilitate more realistic

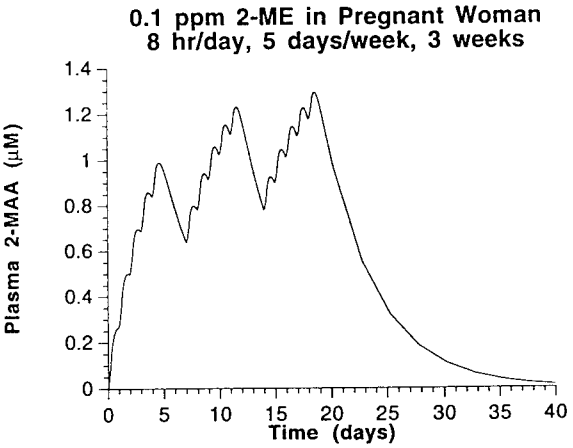


Fig. 11. Simulation of 2-ME inhalation exposure at the OSHA-proposed, much reduced permissible level. This figure depicts the pattern of simulated 2-MAA plasma levels in a pregnant woman who is exposed to 2-ME at 0.1 ppm for 8 h/day for 3 working weeks of 5 days each.

human risk assessments for the developmental toxicity of 2-ME and related compounds. The broader applicability to other chemicals will require careful evaluation and validation with other developmental toxicants.

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Toxicology Letters 82/83 (1995) 549–554

Toxicology Letters

The application of benchmark dose methodology to data from prenatal developmental toxicity studies[1]

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Abstract

The benchmark dose (BMD) concept was applied to 246 prenatal-developmental toxicity (DT) datasets from government, industry and commercial laboratories. Five modeling approaches were used, 2 generic and 3 specific to DT models. BMDs for both quantal and continuous data were compared with statistically derived no observed adverse effect levels (NOAELs) to determine similarities. Quantal (Q) endpoints included litter responses (e.g., one or more dead or malformed implants), and QBMDs were calculated using a Q Weibull (QW) model. Two types of continuous (C) data were modeled, the proportion of implants affected per litter, and the change in fetal weight (both mean and distribution); continuous power (CP) and DT models were used to calculate CBMDs. QBMDs for a 5% change in response ($QBMD_{05}$) were 6-fold lower, on average, than the corresponding NOAEL. $CBMD_{05}$s on average were similar to the corresponding NOAELs, and $CBMD_{05}$s from different models were similar to each other. Including litter size but not threshold improved the fit of the DT models. For fetal weight data, specific cutoff values were used to calculate BMDs that were similar on average to the corresponding NOAELs: (1) changes from the control mean (5% of the mean, 25th percentile of the control distribution, or a decrease of 0.5 standard deviation), and (2) a 5 or 10% decrease in the proportion of fetuses below the 5th or 10th percentile, respectively, of the control distribution. These results support the use of BMDs as providing a more consistent basis for risk assessment than do NOAELs.

Keywords: Benchmark dose; Developmental toxicity; Risk assessment

1. Introduction

Reference dose (RfD) or reference concentration (RfC) estimation for agents that cause developmental toxicity (DT) has been based typically on the determination of no observed adverse effect levels (NOAELs). The NOAEL identifies the largest experimental dose that does not demonstrate a significant adverse response compared to a control group. Uncertainty factors

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[1] The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

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and a modifying factor are applied to the NOAEL to estimate the chronic RfD as well as the RfD for DT (RfD_{DT}) [1]. NOAELs are typically derived by expert evaluation of standard statistical comparisons and biological dose-response considerations. The estimation of statistically derived NOAELs for DT has been investigated using trend tests to derive a no-statistical-significance-of-trend (NOSTASOT) value [2] and these compare favorably with NOAELs derived using the standard approach.

The NOAEL approach for calculating the RfD has been criticized in several ways and an alternative to the NOAEL proposed by Crump [3] is referred to as the benchmark dose (BMD) approach. The BMD approach uses all of the experimental data to fit one or more dose-response curves. The BMD is defined as the statistical lower bound on a dose corresponding to a specified level of risk for quantal (Q) responses. The selected level of risk is in the lower range of the observed data (e.g., 1, 5 or 10%). For continuous (C) data, a level of effect (termed the benchmark effect – BME) must be defined as an adverse change from a control value and the lower bound on dose corresponding to the BME is the BMD. C data may also be transformed to Q data using cutoff values, with BMDs then defined as above.

To determine the general applicability of the BMD approach, several models were applied to both Q and C endpoints from a large number of conventional DT studies. BMDs were derived and compared with statistically derived NOAELs [4–6].

2. Methods

A database containing 246 DT studies with a total of 1825 Q endpoints (dead or resorbed implants, or malformed fetuses) was used for this investigation [2]. Data were from studies conducted in rats, mice or rabbits. Two types of statistically derived NOAELs were calculated for Q endpoints: (1) a QNOAEL for the Q measure of response (i.e., number of litters with one or more affected fetuses or implants), and (2) a CNOAEL for the more continuous measure of response (i.e., proportion of fetuses or implants affected per litter). The data from each study were subdivided into data subsets for a maximum of 9 endpoints or combinations of endpoints (e.g., dead and resorbed, malformed, fetuses with external, visceral or skeletal malformations, and combinations thereof). BMDs were calculated for the same endpoints as were the NOAELs (i.e., QBMD and CBMD, respectively) using 2 generic models, the Q Weibull (QW) model and the continuous power (CP) model [4]. Maximum likelihood estimates (MLEs) and 95% lower bounds for dose corresponding to 3 levels of additional risk (10, 5 and 1%) were calculated. As an example of the notation used, the $QMLE_{10}$ or $QBMD_{10}$ refers to the values derived from the QW model for a 10% additional risk of an affected litter. Similar notations are used for CMLEs and CBMDs calculated using the CP model. Comparisons of the Q and C MLEs and BMDs were made with the corresponding Q and C NOAELs and lowest observed adverse effect levels (LOAELs) for each end point, and QBMD and CBMD comparisons were also made.

Three additional models were used that were specifically designed to account for unique features of data from DT studies [5]. The 3 DT models were applied to a total of 607 endpoints from 141 studies showing significant dose-related effects. These models were termed the RVR model [7], the LOG model [8], and the NCTR model [9]. Models were generalized to account for the correlations among observations in individual fetuses or implants within litters; the potential for variables other than dose, such as litter size, to affect the probability of adverse outcome; and the possibility of a threshold dose below which background response rates are unaltered. MLEs and BMDs were calculated for the probability of a 5% additional risk above controls. An evaluation of model fit was conducted with and without litter size as a covariable and with and without a threshold parameter. χ^2 statistics were used to assess model fit to the observed dose-response data. The variance terms used to define the χ^2 statistic accounted for the correlated (*b*-binomial) nature of the observations. BMDs from the DT models were compared with statistically derived NOAELs, and

BMDs from the 3 DT models were also compared with one another.

A subset of the database, including data from 173 studies, was used for the analysis of fetal weight [6]. Based on preliminary evaluations to determine which definitions of the BME gave BMDs closest to the NOAEL, 6 BMEs for fetal weight were defined. These were (1) a reduction in average litter weight by 5%; (2) a reduction in average litter weight to the 25th percentile; (3) a reduction in average litter weight by 0.5 S.D.; or (4) by 2 S.E.M.; (5) a 5% increase in the proportion of fetuses weighing less than the 5th percentile; or (6) a 10% increase in the proportion weighing less than the 10th percentile. BMDs for the first 4 BMEs were calculated using the CP model; BMDs for the last 2 BMEs were calculated using the LOG model which considered litter size and within-litter correlations. BMD estimates were compared with one another and with the corresponding statistically derived NOAELs for fetal weight data.

3. Results

3.1. Generic models

In general, the QW and CP models used to calculate MLEs and BMDs provided good fits for the dose-response relationships exhibited by the datasets. Goodness of fit tests rejected the fit of the model in a very small percentage of cases (1–4%), and nonconvergence occurred in only 4/1825 cases. In many cases of poor fit, the dose-response pattern was nonmonotonic, with the low dose having a lower response than controls and the mid and high doses showing 100% response.

Table 1 gives selected ratios of BMDs to statistically derived NOAELs. All 3 risk levels of the QBMD tended to be less than the QNOAEL. Of the 3, the $QBMD_{10}$ was closest, on average, to the QNOAEL. In 44% of cases, the $QBMD_{10}$s were within a factor of 2 of the corresponding QNOAEL, 88% were within a factor of 5, and 98% were within a factor of 10. This suggests that the Q measure of response was relatively insensitive, and that QNOAELs tended to be at doses consistent with a 10% or greater risk, while QLOAELs were about 5–7-fold greater. These results have implications for Q data of all types.

For the proportional data, the CNOAEL and the $CBMD_{05}$ were very similar, on average. Ninety-five percent of the $CBMD_{05}$ values were within a factor of 5 of the corresponding CNOAELs, and only 9 endpoints had $CBMD_{05}$s more than 10 times larger than the corresponding CNOAEL. $CBMD_{10}$s in 92% of cases were within a factor of 5 of the corresponding CNOAELs, but tended to be about 1.5 times

Table 1
Ratios of NOAELs to BMDs for DT data

Ratio	Mean	S.D.	Median
Generic models			
$QNOAEL/QBMD_{10}$	2.9	3.9	2.0
$QNOAEL/QBMD_{05}$	5.9	8.4	4.0
$QNOAEL/QBMD_{01}$	29	44	19
$CNOAEL/CBMD_{10}$	0.72	0.44	0.62
$CNOAEL/CBMD_{05}$	1.2	0.88	0.96
$CNOAEL/CBMD_{01}$	4.3	4.5	2.5
DT models			
$CNOAEL/LBMD_{05}$	1.3	1.4	0.97
$QNOAEL/LBMD_{05}$	1.5	2.4	0.96
$NBMD_{05}/LBMD_{05}$	0.96	0.43	0.96
$RBMD_{05}/LBMD_{05}$	1.1	0.35	1.0
$RBMD_{05}/NBMD_{05}$	1.1	0.32	1.1

Q, from QW model; C, from CP model; L, LOG, from log-logistic model by Kupper et al. [8]; N, NCTR, from NCTR model by Kodell et al. [9]; R, RVR, from Rai and Van Ryzin model [7].

larger than the CNOAELs. CLOAELs tended to be closest, on average, to the $CBMD_{10}$. Dividing the CLOAELs by 10 (the default uncertainty factor used when there is no NOAEL) resulted in a value 4–5 times smaller than the $CBMD_{05}$, suggesting that a 10-fold uncertainty factor is too large in many cases.

The QBMDs were almost always less than the corresponding CBMDs for all levels of risk. At a risk level of 10%, the QBMDs were 2–3 times smaller, on average. This difference was attributable to 2 factors: (1) QMLEs tended to be less than the corresponding CMLEs and are dependent on the dose-response pattern, the level of risk, the number of fetuses per litter, and the background rate of response; and (2) the confidence intervals for Q endpoints tended to be wider, resulting in a lower QBMD for a given level of risk. The ratio of the $CMLE_{05}/CBMD_{05}$ in 90% of cases was 1.1 to 2.7, while the $QMLE_{05}/QBMD_{05}$ ratio was 1.6 to 10 in 90% of cases. The greater width of the confidence intervals and the tendency for the QMLE to be less than the CMLE led to a difference of approximately a factor of 3 between the 2 BMD estimates [4].

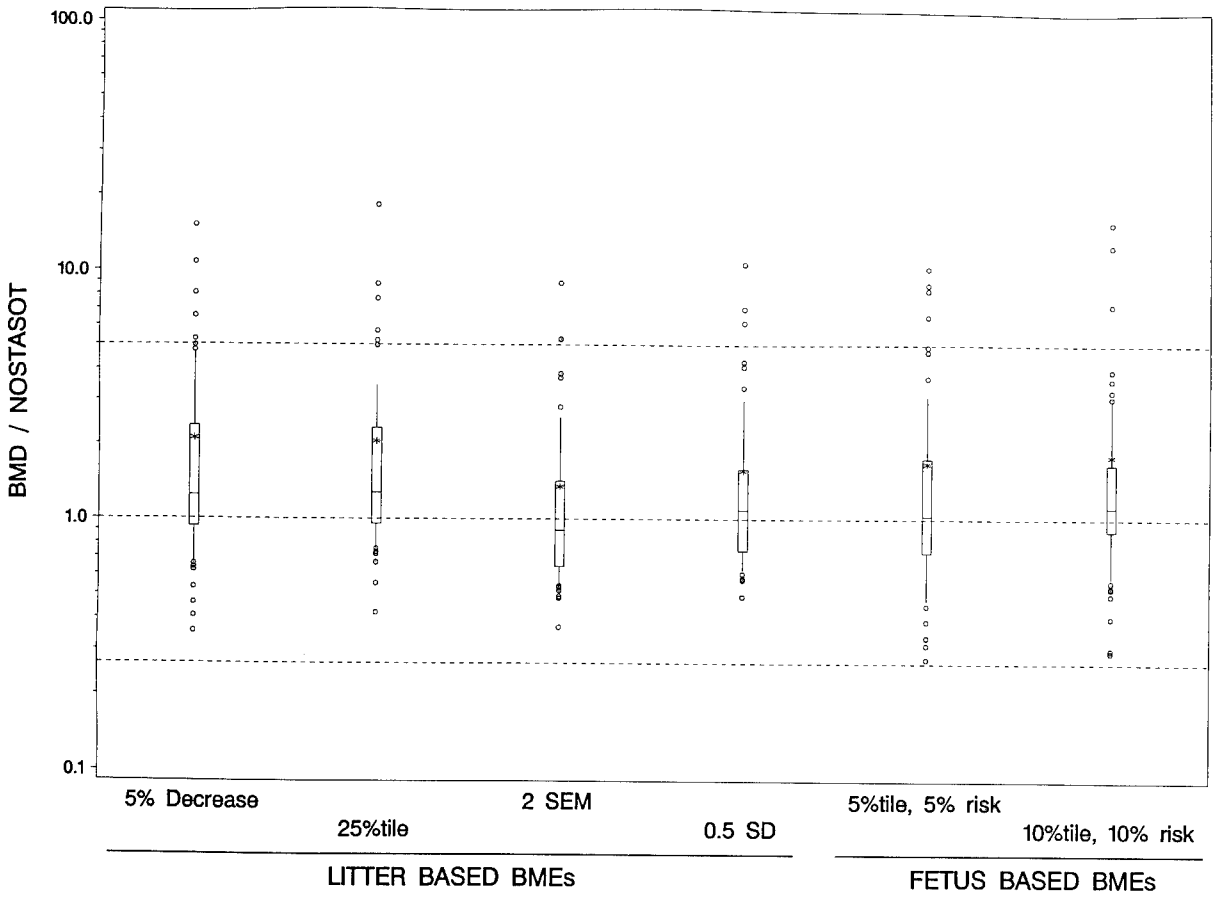


Fig. 1. Distribution of BMD/NOSTASOT (statistically derived NOAEL) ratios for 6 definitions of a BME for fetal weight. For each BME definition the box contains the interquartile range, and the whiskers extend upwards to the 90th percentile and downwards to the 10th percentile. Data points outside that range are indicated by ◦. The horizontal line in each box represents the median value, and the '*' in the box represents the mean (from Kavlock et al. [6]).

3.2. DT models

In general, all 3 DT models fit the data well, with the LOG model providing the best curve fit, in part because it is somewhat more flexible in handling the influence of litter size. Inclusion of litter size in the model often improved the fit, while inclusion of a threshold dose parameter did not. As shown in Table 1, the ratios of the Q and C NOAELs to the LBMDs were approximately 1, on average. The Q and C NOAELs were shown to be similar by Faustman et al. [2]. The ratios of the BMDs from the 3 DT models were also very similar to each other, suggesting that the model used was not critical as long as it fit the data well.

3.3. Modeling fetal weight data

In general, all 6 definitions of the BME used in the modeling approaches resulted in BMD/NOAEL (NOSTASOT) ratios near unity (see Fig. 1). Mean values tended to be higher than median values, suggesting that some BMDs were considerably higher than the corresponding NOAEL. Closer examination of cases where the BMD was 4-fold or more greater than the NOAEL suggested 2 factors of importance: (1) a shallow dose-response curve making statistical estimation of the NOAEL less certain, and/or (2) wide spacing of doses in the study resulting in very conservative NOAELs. Spearman rank correlations showed very high correlations among the 4 litter-based approaches ($r > 0.98$), between the 2 Q approaches ($r > 0.99$), and among the litter-based and Q approaches ($r > 0.95$). BMD/NOAEL ratios were not as well correlated among themselves ($r = 0.58$–0.90), probably due to the design of studies and placement of doses. Use of the 2 S.E.M. BME is cautioned against, since increasing sample size will tend to decrease the S.E.M., also resulting in a decrease in the BMD based on the S.E.M. This feature is inconsistent with a primary advantage of the BMD to reward better experimental design, such as a larger sample size.

4. Conclusions

The studies reported here demonstrate that the use of BMD approaches can be applied routinely to DT data for both Q and C endpoints. In fact, BMD analyses of DT data have been considered recently by EPA for derivation of RfDs in 2 cases, 1 for methylmercury based on Q neurological endpoints [10], and 1 for boron based on fetal weight reduction (unpublished). When proportional values are used, the BMD_{05} is similar, on average, to the NOAEL, while for Q (yes/no) responses, the BMD_{10} is similar to or less than the NOAEL. Given the similarities in the ratios of the BMDs from DT models used, it appears that the choice of the model is not critical as long as it fits the data well. Certain factors known to affect outcome may significantly improve the fit of the model, e.g., litter size, while use of a threshold dose parameter did not seem to improve the fit.

In modeling C data such as fetal weight, it is necessary to define what constitutes an adverse effect (definition of the BME), in this case, a given weight decrement in a litter or fetus. Since no clear definition was available, we chose BMEs that resulted in BMDs near the statistically derived NOAEL, since that has been used in the past to establish lack of an adverse effect. From our evaluations, several choices of the BME and use of the CP model or a DT-specific Q model gave satisfactory results. Theoretically, there are advantages to including both a litter-based and fetus-based BMD approach due to the possibility that there may be a differential distribution in the degree to which fetuses are affected (e.g., a shift in only the lower weight fetuses). However, this did not seem to be the case in the database considered here.

One of the primary advantages of the BMD approach is that it negates having to repeat a study that does not identify a NOAEL. As long as a study can define a LOAEL, it is likely to be amenable to modeling and calculation of a BMD. Kavlock et al. [11] and Weller et al. [12] have demonstrated that study designs which include doses with responses in the range of the BME will improve estimation of the BMD. Use of a BMD approach during the preliminary dose-finding phase may also aid in the design of studies to obtain the most useful data for deriving a BMD.

Additional efforts are needed to examine the BMD approach when defining the critical effect in a study, the application of multivariate models [13], and use among a number of different types of endpoints for overall evaluation of the toxicity of a chemical. Use of the BMD approach is only the first step in moving toward more quantitative approaches that incorporate mechanistic information and provide improved prediction of adverse effects for risk assessment [14].

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Application of benchmark dose risk assessment methodology to developmental toxicity: an industrial view

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Abstract

The U.S. EPA first signalled its intention to use benchmark dose risk techniques in 1991. Subsequently, publication of draft Guidelines for the Risk Assessment of Reproductive Toxicity data indicated the Agency's intention for wide use of the technique. In developmental toxicity experiments, a number of factors need to be considered before attempting benchmark dose calculations, as compared to the conventional NOAEL approach. For example, care in the assessment of potential litter effects (the litter is the unit of such a study) on the data and whether the data are continuous (e.g. foetal body weight) or discontinuous (e.g. specific or grouped developmental defects where the abnormality is present or absent). Two examples of the use of the benchmark dose approach will be made. First, in the analysis of foetal body weight, where a benchmark dose estimate for an agent producing a 5% decrease in mean foetal weight may be calculated from a shift in the distribution of foetal weights between groups, or by conversion of data to reflect changes in the incidence of 'small' pups (i.e. those towards the extreme of the normal range). The second example involves studies conducted on the developmental toxicity of a triazole antifungal. In the first study, the agent was clearly teratogenic, but a NOAEL was not established and thus necessitated a second study. Analysis of benchmark dose estimates (e.g. for % foetuses malformed) from the first study indicated that these were not significantly changed when the data from the second study were combined (i.e. the second study did not aid the risk assessment). The benchmark dose approach has significant scientific and practical advantages over the conventional NOAEL methodology in risk assessments derived from developmental toxicity studies.

Keywords: Benchmark dose, developmental toxicity; Benchmark dose, Triazole; Foetal weight

1. Introduction

In the Risk Assessment guidelines published by the EPA for Developmental Toxicity [1], the Agency proposed for the first time that they intended to use a Benchmark Dose Risk Assessment model for the calculation of virtually safe doses, in comparison to the traditional NO(A)EL/safety factor approach.

The Agency have stated that "most agents causing developmental toxicity in humans alter development at doses within a narrow range near the lowest maternally toxic dose. Therefore, for most agents, the exposure situations of concern will be those that are potentially near the maternally toxic dose range."

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The traditional approach to risk assessment for developmental toxicity involves the establishment of a NO(A)EL (the no observed (adverse) effect level) (i.e. the dose level at and below which no adverse effects are seen) and then applying a safety factor (typically 100) to account for species differences in response and inter-individual variability. Several limitations have been assigned to this type of approach. Firstly, the use of a NO(A)EL focuses on this dose level and does not incorporate information on the shape of the dose-response curve or the variability in the data. Secondly, the NO(A)EL will be higher with decreasing sample size (since variability is not considered), or poor study conduct. Thirdly, the number and spacing of dose levels in a study influence the dose level chosen for the NO(A)EL.

The Agency is therefore proposing the use of an additional quantitative approach for the estimation of dose-response relationships. This involves the estimation of a 'benchmark dose', based on all the data in the study and which corresponds to a small, but measurable increase in the incidence or frequency of an adverse developmental effect and the statistical confidence limits, within the observed range. This is typically a 5 or 10% increase in incidence (ED_{05}; ED_{10}). More usually the benchmark dose is quoted as the lower confidence limit on these increases (i.e. LED_{05})

The 'benchmark dose' offers some advantages over the traditional approach as it is based on all the data. It can be used to calculate margins of safety, or with suitable safety factors, be used to set safe levels of exposure. In some cases a benchmark dose can be determined from studies which do not exhibit a clear NO(A)EL. However, the use of dose-response modelling outside of the experimental range to 'predict' risk at doses below the experimental range is not justified as the shape of the dose-response at low doses cannot be determined by results from a standard animal study or from current knowledge of the mechanism of action of developmental toxicants.

The Central Toxicology Laboratory (CTL) approach has been to develop appropriate mathematical models/methods and define benchmark doses using our own datasets for developmental toxicity studies, including those where repeat studies have been necessary. Benchmark doses were then compared to NOAELs for these datasets to investigate the practical implications of the approach including advantages, disadvantages and pitfalls. Developmental toxicity datasets have a number of special features; typically they are large with 24 litters, 270 foetuses per group. Thus, there can be both litter and foetal effects. A normal study will typically cover multiple endpoints having both quantal (i.e. presence or absence) and continuous variables.

The objectives of the current paper are to explore two examples of datasets illustrating the utility of the benchmark dose approach. Firstly, in the analysis of foetal body weight, this continuous variable requires handling of the data in a different manner from quantal variables [2]. The second example involves two studies conducted on the developmental toxicity of a triazole antifungal. In the first study, the agent was clearly teratogenic, but a NOAEL was not unequivocally established and thus necessitated a second study. Analysis of benchmark dose estimates (e.g. for percentage of foetuses malformed) from the first study indicated that these were not significantly changed when the data from the second study were combined (i.e. the second study did not aid the risk assessment).

2. Example 1

The reduction in foetal weight endpoint was considered appropriate as this provides an objective, sensitive indicator of foetal toxicity, unlike the various other indicators of reduced ossification which can depend on the observer. There was a need for a model of foetal weight as a function of dose. This model should consider factors other than dose which may impact on foetal weight including litter size, size and weight of dam and post-implantation loss.

Risk assessment for a continuous endpoint is more complex than simple quantal changes. A biologically significant manifestation of a reduc-

tion in mean foetal weight can be considered to be an increase in the number of foetuses which are so small that post-natal survival, or development is impaired. A small foetus may then be defined on the basis of a critical weight below which development or survival may be impaired. Notionally, we have taken this weight to be 2 S.D.s below the mean foetal weight of the control population. The number of small foetuses can then be estimated from the distribution function using commercially available computer programs (TERAMOD, TERALOG and TERAVAN [2]). For all dose groups so far studied, the foetal weights are normally distributed about the mean. Using a series of control datasets, an investigation of relationship of litter size to foetal weight indicated that there was a linear relationship, within the normal limits of litter size, for the APfSD (Wistar-derived) rat (Fig. 1). Analysis of a dataset from an agent which did induce a decrease in foetal weight indicated a good dose response relationship (Fig. 2). This agent had also been the subject of a follow up study which also indicated, through the choice of a different dose level in the second study, that the data from the two studies were comparable (Fig. 2) and aided the assessment of the dose response relationship.

A benchmark dose can be calculated based on two alternative approaches. A BMD may be calculated based on a 5% reduction in foetal

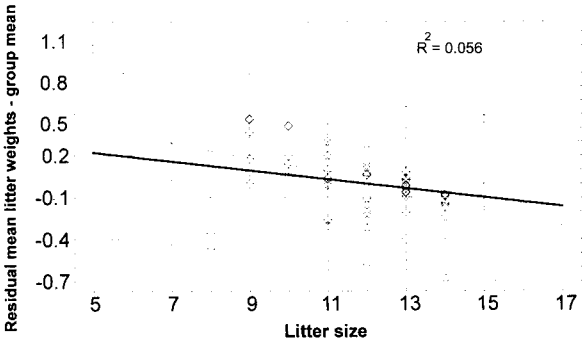


Fig. 1. Effect of litter size on foetal weight for control datasets for the APfSD (Wistar-derived) rat. The solid line represents the regression line with the dotted lines the confidence limits.

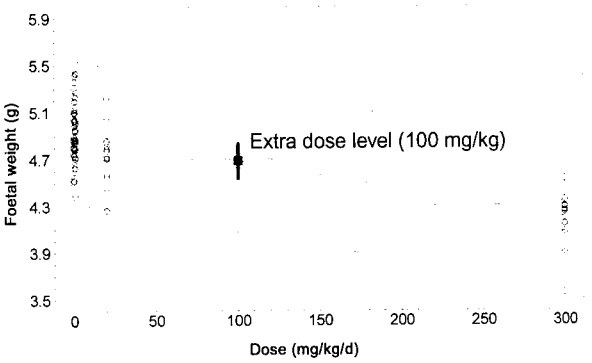


Fig. 2. Dose relationship for the effect of treatment with an agrochemical on foetal weight. The value shown in bold (mean ± S.D.) is for an additional dose level conducted on a subsequent study.

weight using the function of the distribution curves (Fig. 3). Alternatively, it is possible to determine the proportion of small foetuses from the distribution curves and treat the data as discontinuous and thereby calculate the BMD based on a 5% increase in the number of small foetuses (Fig. 4). Either method of calculation yielded very similar results with the LED_{05} for foetal weight reduction of 89 mg/kg/day and a value of 82 mg/kg/day for the increase in the proportion of small foetuses, compared with a NOAEL of 20 mg/kg/day.

It can be concluded that BMD methodology can be applied to continuous data such as foetal weight and that this may be analysed using

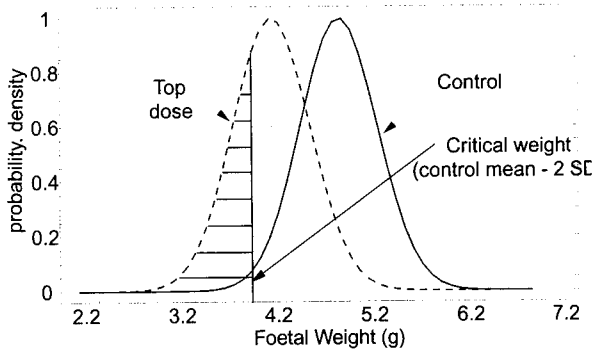


Fig. 3. Frequency distribution curves for foetal weight from a control and a group treated with an agrochemical. The hatched area represents the frequency (or number) of foetuses with a foetal body weight less than 2 S.D.s of the control mean.

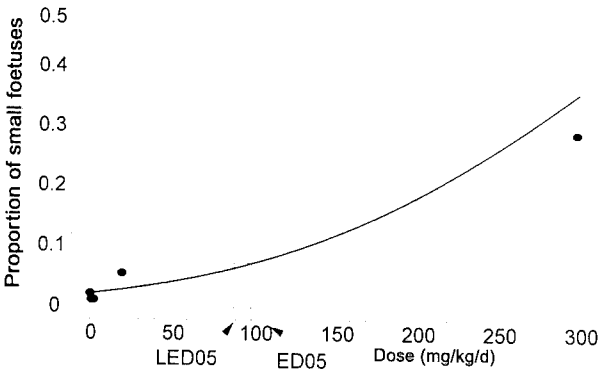


Fig. 4. Representation of benchmark dose calculation (LED_{05}) based on the number of small foetuses from the distribution indicated in Fig. 3.

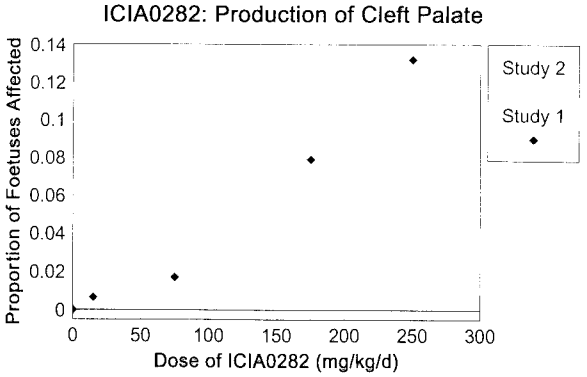


Fig. 5. Dose response relationship for the incidence of cleft palate from two studies conducted with the triazole antifungal ICIA0282.

standard least squares regression models. In addition, litter size has a significant and consistent effect on mean foetal weight. In order to define a BMD, an appropriate degree of change must be selected, for example a 5% reduction in mean foetal weight, based on the smallest measurable and toxicologically significant change. The methods proposed generated BMDs which are conservative and comparable with NOAELs.

3. Example 2

This example illustrates the practical application of the BMD approach with a teratogen. The triazole antifungal ICIA0282[1] induced a wide range of major head defects in a standard rabbit developmental toxicity study, including missing interparietal bones and cleft palate. Dose levels chosen for the initial study were 0, 15, 75, 175 or 250 mg/kg/day. For the induction of cleft palate, a good dose response relationship was obtained, with a single incidence (not statistically significant) at the lowest dose level tested. Such a result indicated that a NOAEL could not be unequivocally ascribed and therefore a repeat study was initiated at dose levels of 0, 2, 7, 12 or 175 mg/kg/day. This study indicated that the cleft palate defect was reproducible at 175 mg/kg/day and established a NOAEL (Fig. 5). Examination of the data from the first and second studies using BMD techniques indicated that although a NOAEL had been determined, the BMD for both studies was almost identical to the first (Fig. 6, Table 1). That is, the second study had determined a NOAEL, but had done little else, in dose response terms. Thus, if the BMD was acceptable to all regulatory authorities, then the second study would not have been necessary with the savings in time, cost and animal use. Moreover, an examination of the

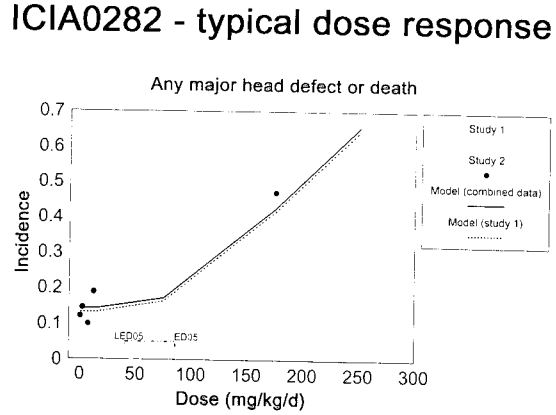


Fig. 6. Typical dose response relationship noted for developmental toxicity induced by ICIA0282. The example given is for any head defect. Dotted lines indicate ED_{05}/LED_{05} based on the modelled data.

[1] The Editor has accepted the use of data for illustrative purposes to indicate how the bencjmarknose technique may be applied.

Table 1
ICIA0282 developmental toxicity in the rabbit: comparison of benchmarks doses and NOAELs

	ED_{05} mg/kg/day)	LED_{05} (mg/kg/day)	NOAEL/LOEL (mg/kg/day)[a]
Foetal death	77	31	75/175
Major skeletal defect	79	30	75/175
Major external defect	77	28	75/175
Any major defect	83	37	75/175
Major head defect	85	40	75/175
Any head defect	60	40	15/75

[a] NOAEL based on Fishers exact test ($P < 0.05$).

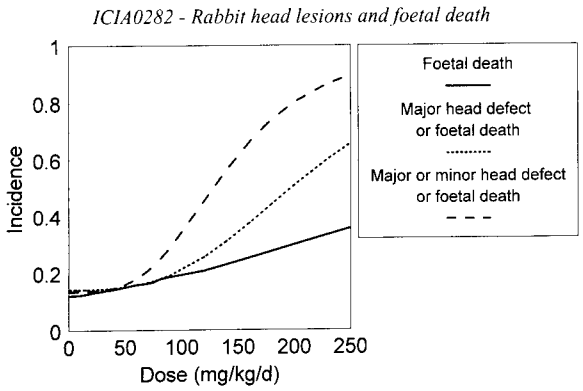


Fig. 7. Dose response relationship for the incidence of a number of indices of developmental toxicity from two studies conducted with the triazole antifungal ICIA0282.

BMD for other endpoints on the studies illustrated the 'classic' dose response curves for a teratogen (Fig. 7). That is different dose response relationships (and hence BMDs) may be derived depending on which aspects of developmental toxicity are modelled with clear differences obtained when data for any specific foetal head defect are compared to those obtained for malformation or death. This example typifies the overlapping dose response relationships for an agent of this type and how choice of dose levels can mask certain developmental effects.

4. Overall conclusions on the advantages of the benchmark dose methodology

A number of clear conclusions can be drawn on the utility of the benchmark dose approach even from the limited numbers of datasets presented in this paper. These may be summarised as:

1. Benchmark doses (BMDs) are a practical alternative to NOAELs for most developmental toxicity datasets, although care needs to be taken with those datasets exhibiting a 'hockey stick-type' dose response and where very low incidence observations are being modelled.
2. BMDs use data from **all** dose levels employed on a study and therefore are a better reflection of the whole of the dataset.
3. BMDs are values that are **within the experimental range** of the study.
4. BMDs are less sensitive to choice of dose levels than the NOAEL.
5. Use of BMD techniques may remove the need for the repeat of some developmental toxicity studies.
6. A BMD calculated on the basis of an LED_{05} was usually comparable with NOAEL.
7. The BMD and the NOAEL may be used to express 'potency', or to derive safe levels for human exposure, but regulators may apply different safety factors.
8. The appropriate use of BMD techniques in the regulatory arena should reward good experimentation.

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Toxicology Letters

In vivo ESR measurements of free radical reactions in living mice

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Abstract

In vivo ESR measurements were carried out to estimate free radical reactions in living mice using nitroxyl radicals as probes. The ESR signal of nitroxyl radical which was intravenously or intramuscularly injected to living female ddY mice decreased gradually by reducing to the corresponding hydroxylamine. The reduction rate was enhanced by oxidative stress, and pre-treatment of antioxidants suppressed the enhancement of signal decay. Oral administration of carbon tetrachloride enhanced signal decay in upper abdomen but not in thorax. These results indicated that free radicals, which can reduce nitroxyl radical, were produced in the upper abdomen by oral administration of carbon tetrachloride.

Keywords: ESR; Free radical; Oxidative damage; Active oxygens; Carbon tetrachloride

1. Introduction

Free radicals such as active oxygen species and nitric oxide are believed to be highly essential and functional compounds in various biological systems and toxicological phenomena, and numerous investigations have been made to detect active oxygen species and to clarify their role in biological phenomena. However, most reports were of in vitro experiments and few of in vivo investigations. Recently, a low-frequent ESR spectroscopy has been developed and enabled non-invasive in vivo measurement of radicals in whole animals [1]. Despite the great possibility, ESR measurement of in vivo radical generation in living animals has rarely been reported because of poor sensitivity of the in vivo ESR spectrometer. ESR signals of nitroxyl radicals are susceptible to oxygen concentration, to active oxygens, and to biological redox systems. This indicates that a combination of in vivo ESR spectrometer with nitroxyl radicals as probes may provide valuable information about biological function of free radical reaction, including generation of active oxygens and activity of redox systems. We have studied the in vivo free radical reactions using the nitroxyl radicals which have been administered intravenously [2–5], intramuscularly [3], intraperitoneally [6], and transtracheally to mice [7,8]. The results were reduction of nitroxyl radical and the revelation that the rate of reduction should depend on

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physiological and pathological conditions such as aging and oxidative stress. In the present paper, we also applied in vivo ESR measurements to carbon tetrachloride induced injury of living mice to estimate in vivo free radical reactions using nitroxyl radicals as probes.

2. Materials and methods

3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) was purchased from Aldrich Chemical Co. and was dissolved in isotonic solution. Female ddY mice (3–4 weeks old, 15–20 g body weight) were used throughout this study. Mice were anesthetized by intramuscular or intraperitoneal injection of pentobarbital (50 mg/kg) and fixed on a hand-made Teflon holder.

Hypo- and hyper-oxia experiments were performed by exposing mice to an atmosphere of N_2–O_2 mixture (12%, 20% and 80% O_2 in N_2) for 45 min before ESR measurement. The spin-probe was intravenously administered, and the ESR spectrum was observed under the above atmosphere. Ischemia-reperfusion of mouse's thigh was carried out by modifying the method of Oyanagui et al. [9]. Occlusion was done by tying the base of the femoral muscle with a thread for 20 min, and followed by reperfusion. Spin-probe was administered to femoral muscle of mice 1 min before reperfusion. Carbon tetrachloride was suspended in 1% of carboxy methyl cellulose solution, and one-third of LD_{50} of carbon tetrachloride (3.3 g/kg body weight) was orally administered to mice. After administration of spin-probe intravenously, the ESR spectrum was measured at various periods after the administration.

ESR spectra at different domains from head to tail were obtained with an in vivo ESR spectrometer (JEOL, JES-RE-1L or -3L). The microwave frequency was 1.1–1.3 GHz and the power was 1.0–5.0 mW. The amplitude of the 100 kHz field modulation was 0.2 mT. The external magnetic field was swept at a scan rate of 5 mT/min.

3. Results and discussion

Fig. 1a shows typical ESR spectra from the upper abdomen of a mouse with carbamoyl-PROXYL administered into the tail vein. Three sharp lines were observed with regular noise due to respiration. The hyperfine structure and the peak height ratios of carbamoyl-PROXYL coincided with those of the probe dissolved in saline at a concentration of less than 10 mM, suggesting that the spin-probe should exist as a free monomer in veins at the hepatic domain. Quite similar spectra were also observed at head, chest, and lower abdomen after intravenous administration.

The ESR signal of nitroxyl radicals decreased gradually in living mice. Fig. 1b shows a semilogarithmic plot of the peak heights in the upper abdomen after intravenous administration of carbamoyl-PROXYL. The plot was a straight line till at least 10 min, indicating that signal decay after i.v. injection should obey first order kinetics.

Table 1 demonstrates decay constants of carbamoyl-PROXYL at the head and abdomen of mice that were exposed to different oxygen concentrations [4]. The decay constant under 12% oxygen was significantly larger than that under 20% oxygen at both domains. We previously reported that nitroxyl radical loses its paramagnetism more rapidly by incubating with microsomes under hypoxic condition [10,11]. A hypoxic condition may also favour reduction of nitroxyl radicals in living mice. The decay constant of carbamoyl-PROXYL in the abdomen under 80% oxygen was significantly greater than that under 20% oxygen ($P < 0.001$). Active oxygen species such as O_2^-, $\cdot OH$, and H_2O_2 are reported to be generated in the liver under hyperoxia [12], and the nitroxyl radical loses its paramagnetism by interaction with active oxygen species [13]. In fact, the pre-load of antioxidants such as Trolox, uric acid, and glutathione retarded the enhancement of signal decay under hyperoxia (Table 2), and their retardations corresponded to those estimated with TBA-reactive substances [5]. The i.p. administration of

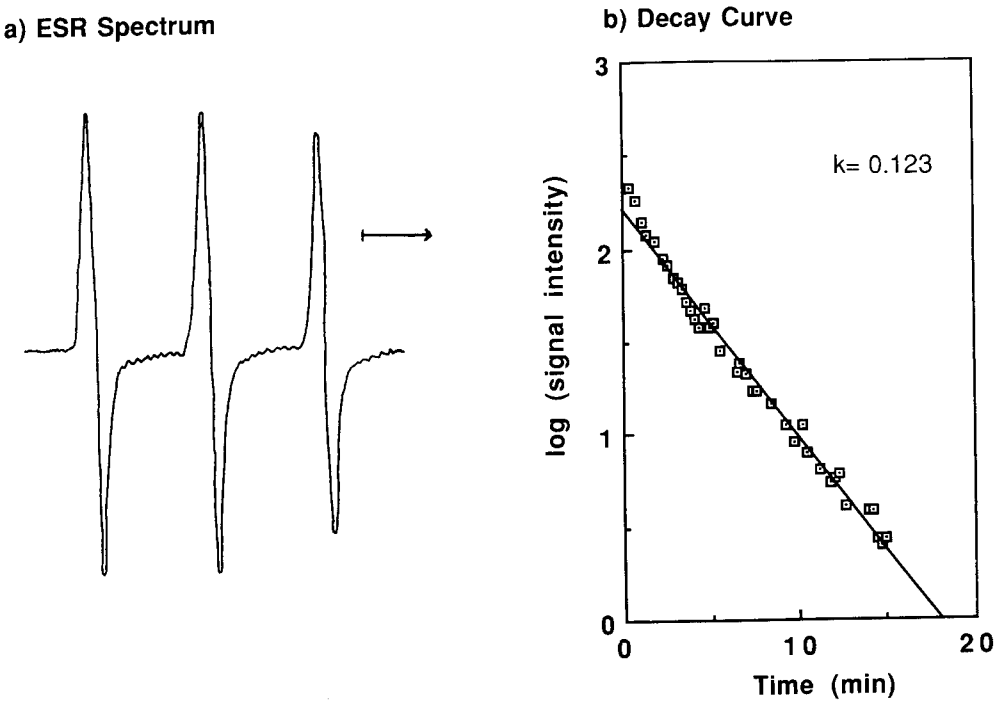


Fig. 1. ESR spectrum and signal decay curve of carbamoyl-PROXYL in the upper abdomen of a mouse after i.v. administration. (a) A solution of carbamoyl-PROXYL (280 mM, 50 μl) was injected into a tail vein of an anesthetized female ddY mouse and the ESR spectrum in the upper abdomen was observed with an ESR spectrometer as described in the Materials and methods. Arrow indicates the direction and amplitude (1.0 mT) of external magnetic field. (b) A peak height $h(+1)$ of triplet signals was plotted against time after injection.

Table 1
Decay constant of carbamoyl-PROXYL under various O_2 concentration in whole mice (per min) [4]

O_2 Concentration	Abdomen	Head
12%	0.119 ± 0.011^{a}	0.103 ± 0.008^{b}
20%	$0.095 \pm 0.015^{a,c}$	0.073 ± 0.005^{b}
80%	0.147 ± 0.019^{c}	0.071 ± 0.007

Decay constants are presented as mean ± S.E. over 5 or 6 experiments.
[a,b,c] The difference between two groups is significant with $P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively.

ascorbic acid at a dose of 10 mg/kg body weight did not show any effect in vivo, although it can reduce nitroxyl radical quickly in vitro.

Ischemia-reperfusion also enhanced the in vivo signal decay [3]. Spin-probe was first injected into the left thigh and ESR spectra were measured till any signal became undetectable. Then the same amount of the probe was injected into the right thigh with and without prior treatment of ischemia-reperfusion. Again, the clearance constant of the right thigh was measured, and the ratio of the clearance constant of the right thigh to those of the left one was used to estimate the effect of ischemia-reperfusion on the radical reduction. The ratio in the group treated with ischemia-reperfusion (0.79) was significantly larger than that without ischemia-reperfusion (1.54), and the increment of the ratio was inhibited by the treatment of superoxide dismutase (SOD) or allopurinol, suggesting that generation of O_2^- contributes to the signal reduction by ischemia-reperfusion injury of mouse thigh.

Oral administration of carbon tetrachloride also enhanced the signal decay of nitroxyl radical

Table 2
Effect of various antioxidants on decay constants for carbamoyl-PROXYL in abdomen under normoxia and hyperoxia [5]

	Dose (mg/kg)	20% O_2	80% O_2
Control 1		0.102 ± 0.012^{a}	$0.138 \pm 0.014^{b,d}$
Control 2		0.105 ± 0.015	0.132 ± 0.012^{c}
Trolox	1	0.106 ± 0.010	0.114 ± 0.015^{c}
Uric acid	10	0.109 ± 0.011	0.104 ± 0.021^{b}
Glutathione	10	0.115 ± 0.009^{a}	0.109 ± 0.014^{d}
Ascorbic acid	10	0.105 ± 0.016	0.135 ± 0.008

Decay constants are presented as mean ± S.E. over 5 or 6 experiments. Mice in control groups 1 and 2 were administered with vehicles of 0.2 ml of saline and saline containing 1.4% ethanol, respectively.
[a,b,c,d] The difference between two groups is significant with $P < 0.1$, $P < 0.1$, $P < 0.01$, and $P < 0.005$, respectively.

at the upper abdomen of mice but not at the thorax. This enhancement was predominant 30 min after administration. These results indicates that the oral administration of carbon tetrachloride generates some free radical species which have the capability to reduce nitroxyl radical.

In conclusion, we have demonstrated that in vivo ESR spectroscopy with nitroxyl radical as a probe makes it possible to estimate free radical reactions in living animal non-invasively, since the nitroxyl radicals are susceptible to both biological redox state and active oxygen species. It was found that the in vivo signal decay of nitroxyl radicals is influenced by physiological and pathological phenomena such as inspired oxygen concentration, ischemia-reperfusion injury, and oral administration of carbon tetrachloride. The present paper strongly suggests that in vivo ESR measurement with nitroxyl radical as a probe should be a very useful technique to estimate the influence of antioxidants on biological radical reactions in the living body.

Acknowledgements

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Mass spectrometric approaches to molecular characterization of protein-nucleic acid interactions

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Abstract

The recent development of 'soft' ionization-desorption methods has lead to a breakthrough for the mass spectrometric analysis of biomacromolecules such as proteins and nucleic acids. In particular, the feasibility of electrospray-ionization mass spectrometry (ESI-MS) for the direct characterization of non-covalent supramolecular complexes is opening new analytical perspectives. Examples hitherto analyzed by ESI-MS include enzyme-substrate and -inhibitor complexes, homo- and heterodimers/trimers of leucine zipper polypeptides, and several other DNA- and RNA-binding proteins. Furthermore, the characterization of double-stranded and higher-order oligo- and polynucleotide complexes by negative-ion ESI has been demonstrated. Ions specific of non-covalent protein and oligonucleotide complexes can be selectively dissociated by changing the solution conditions and by increasing the desolvation potential. These results form the basis for the molecular characterization of protein-nucleotide interactions, thus complementing protein-chemical approaches, and other methods of structure determination.

Keywords: Supramolecular protein complexes; Electrospray-ionization mass spectrometry; Leucine zipper polypeptides; Double-stranded oligonucleotides; Protein-nucleotide interactions

1. Introduction

The direct mass spectrometric analysis of biomacromolecules has experienced a breakthrough in recent years by the development of efficient 'soft' desorption ionization methods such as fast atom bombardment (FABMS), 252-Cf-plasma desorption (PDMS), and particularly, electrospray-ionization (ESI-MS) and matrix-assisted laser desorption-ionization (MALDI-MS) [1]. Intact biopolymer molecular ions have been obtained most successfully by ESI- and MALDI-MS (up to several 100 kDa for proteins) [2,3]. In combination with specific chemical or mass spectrometric fragmentation, these methods have found already broad application to *primary* structure characterization such as sequence determinations and covalent post-translational structure modifications [4]. In contrast to MALDI-MS which produces essentially denatured protein molecular ions, multiple charged molecular ion series and distributions of 'native' protein

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states in solution are obtained by ESI-MS (e.g. $[M + nH]^{n+}$), and have been shown to provide information about solution conformation and tertiary structures [2,5]. Moreover, the recently discovered feasibility of ESI-MS in the direct analysis of intact supramolecular complexes is leading to new analytical perspectives, particularly the study of non-covalent protein interactions [5,6].

A number of specific non-covalent complexes of biopolymers have been analyzed by ESI-MS already, such as enzyme-substrate or -inhibitor complexes, protein quarternary structures and double-stranded oligonucleotides [6–11]. These successful initial model studies have prompted our interest and provide the basis for a systematic evaluation of analytical preconditions and applications of the ESI-method to the characterization of protein-polynucleotide interactions. In this article, recent studies in our laboratory on (1) leucine zipper polypeptides and other DNA- and RNA-binding proteins, and (2) protein- and nucleotide-interactions of the elongation factor-Tu (EF-Tu) from *T. thermophilus* are summarized. The current results indicate ESI-MS as an efficient tool for the direct identification of protein-nucleotide interactions at the molecular level, hence extending and complementing chemical and spectroscopic methods for their structural characterization.

2. Results and discussion

2.1. Macromolecular ion formation and preconditions for analysis of non-covalent protein complexes by ESI-MS

First successful analyses of non-covalent complexes by ESI-MS have led to an increasing number of model studies, and applications to supramolecular biopolymer structures as well as synthetic self-assembly systems [5,12]. Furthermore, this unexpected feature of the electrospray method has stimulated the detailed evaluation of the ion formation mechanism(s) by field-induced desolvation from solution [13]. Although the pathways of desolvation and macromolecular ion-formation have not yet been elucidated sufficiently well to derive direct information about tertiary structures, useful correlations between solution structures and multiple charged ions [7,10] have been obtained. A qualitative differentiation of charge state distributions ('charge structures' [5]) has been observed for 'native' protein structures compared to spectra of irreversibly denatured proteins, e.g. upon thermal and solvent-induced denaturation [7]. An illustrative example demonstrating the correlation of solution structure and molecular ion (charge) pattern has been the pH-dependent dissociation and reconstitution of myoglobin, one of the first non-covalent protein complexes detected by ESI-MS [6]. Dissociation by acidification yielded characteristic high charge-state ions of the apoprotein, whereas renaturation at pH 6 provided the reconstituted intact heme-protein ions [10]. Furthermore, structural states of proteins have been probed by deuterium exchange in solution and by comparative analyses of chemically modified proteins yielding results consistent with NMR data [7,14,15]. Although little is yet known to which extent solution structures are reflected by charge distributions of molecular ions these results appear to fulfil one prerequisite for detecting supramolecular protein complexes.

Preconditions and parameters of ESI-MS which have been employed for the characterization of non-covalent protein complexes are summarized in Table 1. Most important is the evalua-

Table 1
Preconditions and ESI-MS parameters for identification of non-covalent protein and polynucleotide complexes

Criteria for identification of supramolecular complex	ESI-MS parameters/dissociation by
◆ Specific complex stoichiometry/equilibrium of complex components	Increase of declustering voltage (~20–100 V). Increase of interface temperature. Change of solution stoichiometry.
◆ Competition of complex components	Chemical modification/specific mutation of components.
◆ Specificity of solution conditions	Change of pH; buffer; concentration.
◆ Gas-phase stability of complex ions	Increased declustering voltage. Collision-induced dissociation.

tion of experimental conditions that provide (1) the differentiation of *non-covalent* complexes and *covalent* adducts, and (2) the distinction of possible unspecific aggregation products or 'cluster' ions. The latter artefact ions – although observed in most desorption-ionization MS methods [5] – do not appear to be significant under ESI-MS conditions [7]. A critical step for the detection of non-covalent interactions is the desolvation of macro-ions supported by a small declustering (ca. 10–100 V) potential between the inlet capillary tip and skimmer (repeller) electrode (counter-electrode-skimmer potential, ΔCS); the lower ΔCS, the higher is the chance that non-covalent complexes survive the desolvation process as illustrated by the observation of water-solvated polypeptide ions at 'incomplete' desolvation conditions [7]. A number of experimental parameters may be required to ascertain specific binding structures, such as competition studies of complex components or changing solution conditions (pH, concentration). Non-covalent protein complexes analyzed under these conditions encompass different types of ligands and binding (such as ionic and hydrogen-bond interactions), with dissociation constants ranging from 10^{-6} to 10^{-11} M [6]. These model studies suggest broad application potential of ESI-MS for the study of protein- and nucleotide-interactions.

2.2. Identification of leucine zipper complexes

Several leucine zipper classes of proteins have attracted interest in the last years because of their function in regulating transcription by specific DNA recognition [16]. The leucine zipper structural motif, consisting of a repeating 4-3 heptad of hydrophobic and non-polar residues with Leu dominating at position 4, was first detected in one of the simplest DNA-binding structures, the basic region leucine zipper (bzip) where it mediates the dimerization of 2 basic regions to a DNA-binding site [17]. Probably the best known leucine zipper is the C-terminal domain of the yeast transcription-activator, GCN4-p1 which thus seems to be an appropriate model system for studying corresponding protein and nucleotide complexes by ESI-MS [18,19]. As shown by the crystal structure, GCN4-p1 assembles to a dimeric parallel coiled coil in which hydrophobic interactions between the α-helical polypeptide chains are the dominant stabilizing forces (see structure in Fig. 1).

Systematic studies by ESI-MS were carried out on a series of synthetic leucine zipper polypeptides, based on the sequences of naturally occurring coiled coils [16]. The ESI spectrum of GCN4-p1 at low declustering potential (ΔCS, 10 V) is shown in Fig. 1a. The direct identification of a dimeric complex, (M_2, M_r 8078 Da) is provided by the 5 + charged macro-ion at m/z 1617 ((2 × 4039 + 5)/5 = 1616.7), whereas the ions at m/z 1347 and 1010 can originate from both the monomer and/or dimer $(M)^{3+}/(M_2)^{6+}$,

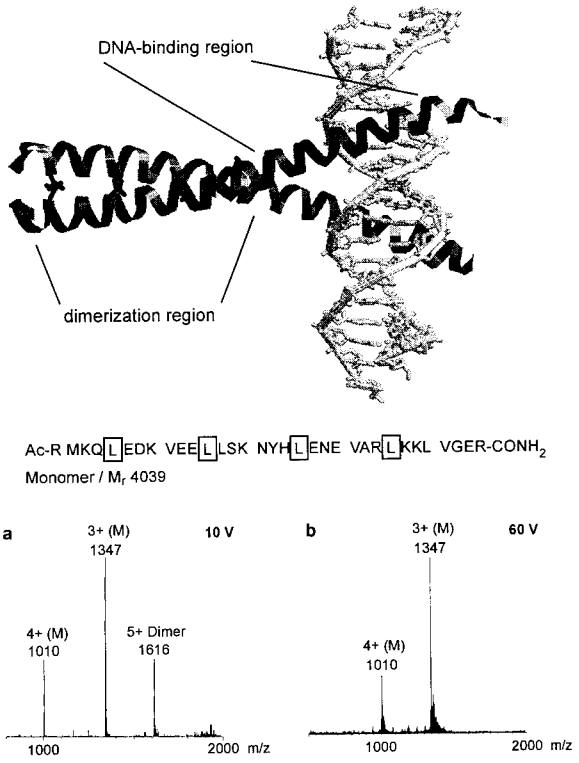


Fig. 1. ESI mass spectra of leucine zipper polypeptide GCN4-p1 at 10 (a) and 60 V (b) repeller declustering voltage (ΔCS). The dimeric structure is only observed at ΔCS = 10 V by the 5 + charged molecular ion. Analysis was performed by injection of 5 μl 50 μM peptide in 2 mM NH_4OAc:methanol (9:1), pH 6.0. The top insert shows the coiled-coil GCN4-p1 crystal structure, complexed with the complementary oligonucleotide [17].

and $(M)^{4+}/(M_2)^{8+}$, respectively. Generally, unequivocal identification of an oligomer composed of identical polypeptide chains is obtained by a molecular ion with a non-integer charge number, when divided by the number of complex components, i.e. *charge of molecular ion/number of chains ≠ integer number*. Hence leucine zipper homodimers are characterized by all odd-charged ions. The peak of the $[M + 5H]^{5+}$ ion of the dimer completely disappears upon dissociation at higher ΔCS (60 V; Fig. 1b) whereas the 3 + and 4 + charged ions of monomeric GCN4-p1 remain unchanged which confirms the non-covalent nature of the complex.

Several model leucine zippers based on the sequences of natural coiled coils have been designed, one of which ('coil-Ser') has been crystallized recently and shown to form a 3-stranded coiled coil [20]. In the ESI mass spectrum of a synthetic peptide, LZ, whose sequence is identical with that of coil-Ser except for Trp at position 2 and Ser at position 14, the formation of a trimer was identified by an $(M_3)^{5+}$ ion at m/z 1986 whereas no dimer $(M_2)^{5+}$ was observed [21]. The triple-stranded complex of LZ is in agreement with the crystal structure of 'coil-Ser' and was confirmed by sedimentation equilibrium analysis [21]. Moreover, direct information in equilibria between dimers and trimers and the formation of homo- and heteromeric complexes was obtained by ESI-MS in hybridization experiments of LZ peptides containing different sequence variants. As an example, Fig. 2 shows corresponding spectra of a mixture of LZ and LZ(12A) containing an Ala-substitution in position 12 that destabilizes the coiled coil. Ions characteristic due to the heterodimer, AL^{4+}, and both possible hetero-trimers $(LZ)/(LZ12A)_2$ and $(LZ)_2/LZ12A$ were identified, in addition to the homomeric complexes. In contrast to the low

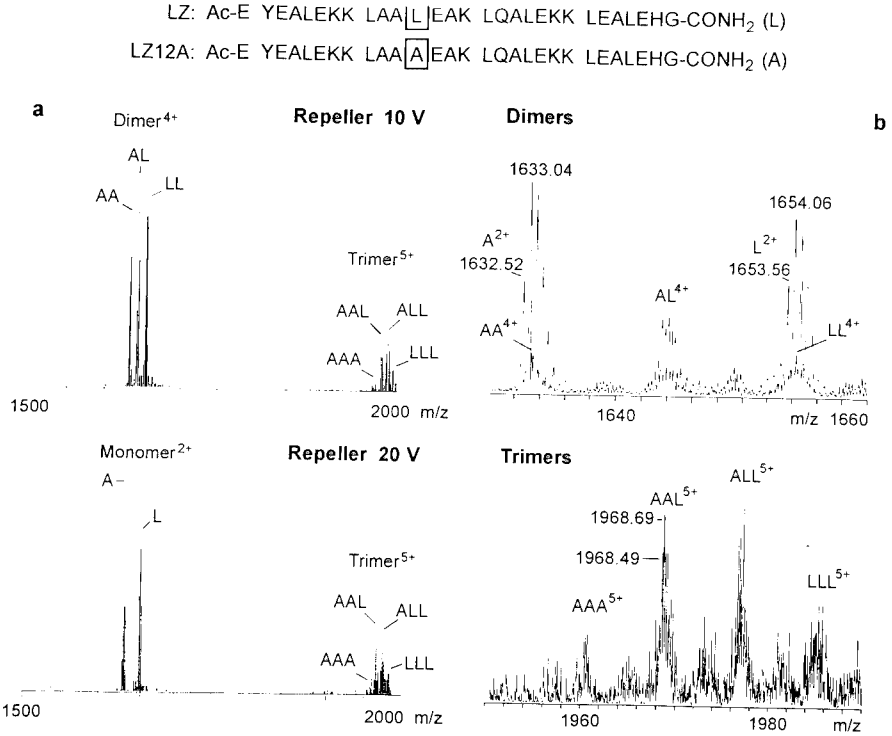


Fig. 2. ESI mass spectra of dimeric and trimeric heteromers obtained from equal volumes of 0.1 mM solutions of peptides LZ and LZ12A. (a) Spectra of dimeric and trimeric complexes on a quadrupole mass spectrometer at ΔCS = 10 and 20 V (bottom spectrum); (b) spectrum of dimers (top) and trimers (bottom) on a magnetic sector instrument, showing isotopic resolution of 4 + charged dimeric and 5 + charged trimeric ions.

resolution ESI spectra obtained with a quadrupole mass analyzer (Fig. 2a), unequivocal assignment of dimeric and trimeric complex ions was achieved on a double focusing magnetic sector instrument that permitted isotopic resolution (Fig. 2b). Thus, a direct differentiation of the 4+ charged homodimer ion within the 2+ charged monomer was provided by the mass differences (Δm, 0.25 amu) of isotopic multiplets at m/z 1633 and 1654. Likewise, correct isotopic resolution (Δm, 0.2 amu) was observed for the trimer $(M_3)^{5+}$ ions. With the ESI-MS method it was even possible to demonstrate that hybridization of GCN4-p1 and LZ peptides produced small amounts of both dimeric and trimeric heteromers. Furthermore, fluorescence quenching experiments confirmed the formation of coiled coil heteromers in solution, and excluded ESI-MS artefacts.

Dimeric complexes of several other DNA- and RNA-binding proteins have been recently identified by ESI-MS (Table 2), including the hydrophobic, lipid-binding lung surfactant protein SP-C [22]. These results lend promise to the ESI method as a tool for detecting even weak (hydrophobic) interactions in nucleotide-binding proteins. However, a significant problem encountered in concentration- and pH-dependent studies is the strong tendency of leucine zippers to adsorb at polar, fused-silica sample delivery capillaries, leading to partial dissociation of complexes [10,19]. Due to this limitation, quantitative information on thermodynamic stabilities on homo- and heteromeric assemblies cannot be obtained at present. The proportions of complex ions could be significantly increased recently by using pre-coated (polyacrylamide) capillaries [21], thus resulting in a rough correlation of molecular ion abundances of dimers and trimers with the stability of the different coiled coils. A further problem is the general, relative reduction of charge states of multiple charged ions for protein complexes, leading to increasing instrumental demands in the detection of high-mass (low charge state) complex ions [10].

2.3. Identification of double-stranded oligonucleotides

In contrast to the already extensive work on polypeptides, negative-ion ESI-MS of oligo- and polynucleotides has been found significantly more difficult with regard to obtaining a homogeneous, poly-phosphate backbone and composition of counter ions [9]. Hence, the purification problems, e.g. from contaminating alkali salts, still present some limitations to the molecular weight range amenable. Best results have been obtained at present with aqueous poly-ammonium salts which have permitted the detection of homogeneous multiple charged M^{n-} ion series in sequences of up to ~80–100 bases [5].

Despite these yet existing limitations, identification of intact double-stranded oligonucleotides has been reported in recent studies [8–10]. The ESI spectrum of the duplex 24-mer palindromic recognition sequence of the GCN4 leucine zipper (GCN4-U/-L), prepared by hybridization in ammonium acetate, is illustrated in Fig. 3 in comparison to a single-strand sequence. Unequivocal identification of the duplex is obtained (as noted above) by the specific *odd-charged* ion $[M-9H]^{9-}$, whereas the masses of the 8-charged duplex ion and the 4-charged monomer ions would not be resolved at the resolution of the quadrupole analyzer employed. However, the charge state distributions in Fig. 3b suggest the nearly quantitative formation of the duplex which was ascertained by independent dissocia-

Table 2
Identification of supramolecular complexes of polynucleotide-binding proteins by ESI-MS

Protein	Mol. wt.	Function/localization	Supramolecular association	Complex identified
GCN4-p1	7165	Transcription activator	DNA binding	Dimer
LZ	~3400	Leucine zipper peptides		Dimer, trimer
L7	12 460	Autoantigen	RNA binding	Dimer
SAF-A	25 100	Nuclear matrix protein	DNA binding	Dimer

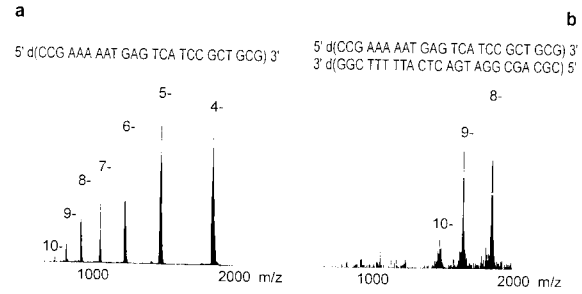


Fig. 3. Negative ion ESI mass spectra of (a) single-strand 24-mer oligonucleotide GCN4-U; (b) double-stranded GCN4-U/-L hybridization product. Solvent, 5 mM NH_4OAc:acetonitrile, 9:1 (pH 6.0).

tion studies and electrophoresis. Furthermore, successful studies of several synthetic oligonucleotides with different sequence/base compositions (see examples in Table 3) showed remarkable stabilities of the duplex forms under ESI conditions relative to leucine zipper polypeptide complexes. These results suggest the possibility for determining duplex melting temperatures using ESI-MS which has been addressed in first model studies [23].

2.4. Characterization of nucleotide complexes of EF-Tu/EF-Ts

The successful characterization of both supramolecular polypeptide and protein complexes and of double-stranded (and higher-order [7]) oligonucleotides appears to provide, in principle, the basis for directly probing protein-nucleotide interactions in solution under ESI-MS conditions. As noted above, possible effects on charge structures of proteins upon polar interaction with a nucleotide backbone are still unknown, as are the resulting multiple charged ions detectable by ESI-MS. Initial model studies on leucine zipper peptide complexes with complementary oligonucleotides did not yield detectable high charge-state ions within a limited mass range (m/z 2000), indicating the requirement for using suitable high mass analyzers (such as time-of-flight instruments) for detecting complex ions of low charge numbers.

However, the suitability of ESI-MS to analyze polar nucleotide complexes in proteins could be demonstrated recently in studies to characterize the nucleotide and protein binding regions in the EF-Tu from *T. thermophilus* [24]. The transition of EF-Tu from an 'inactive' GDP, to the GTP binding form upon interaction with the nucleotide exchange factor EF-Ts was characterized by selective chemical modification and by direct mass spectrometric analysis of the EF-Tu/-Ts and GDP complexes [25]. This study provided the identification of Lys residues for distinct nucleotide binding regions (see Fig. 4). As an example, ESI spectra of free and GDP-bound forms of EF-Tu are compared in Fig. 4 which revealed a homogeneous ion series of a 1:2-EF-Tu/GDP complex, under the conditions em-

Table 3
Examples of double-stranded oligo-deoxynucleotide hybridization products identified by negative-ion ESI-MS

Oligonucleotide[a]	Mol. wt. (D)[b]		m/z	Duplex ions[c]	
	Single strands	Duplex		Charge state[d]	Mol. wt.[e]
dC_4G_4	2411/2411	4822	1607	3	4824
$d(T)_8/d(A)_8$	2371/2443	4814	1624	3	4872
$d(T)_{10}/d(A)_{10}$	2980/3070	6050	1209	5	6052
d(20mer) I/II	5827/6410	12237	2446[f]	5	12297
GCN4-L/-U	7360/7347	14707	1638	9	14714

[a] Oligonucleotide sequences: dC4G4, self-complementary 5'-dCCCCGGGG-3'; d(20mer-I/-II), 5'-dCCTTCCTCCCTCTCTCCTCC-3', 3'-GGAAGGAGGGAGAGAGGAGG-5'; GCN4-L/-U, 5'-dCGCAGCGGATGAC-TCATTTTTCGG-3', 3'-dGCGTCGCCTACTGAGTAAAAGCC-5'.
[b] Monoisotopic molecular weights without counter-ions.
[c] Duplex ions observed as $[M - nH]^{n-}$.
[d] Most abundant charge states observed within a mass range of approximately 2000.
[e] Average molecular weight determined from duplex ions.
[f] Duplex ion obtained with extended mass range quadrupol mass analyzer; see Ref. [9].

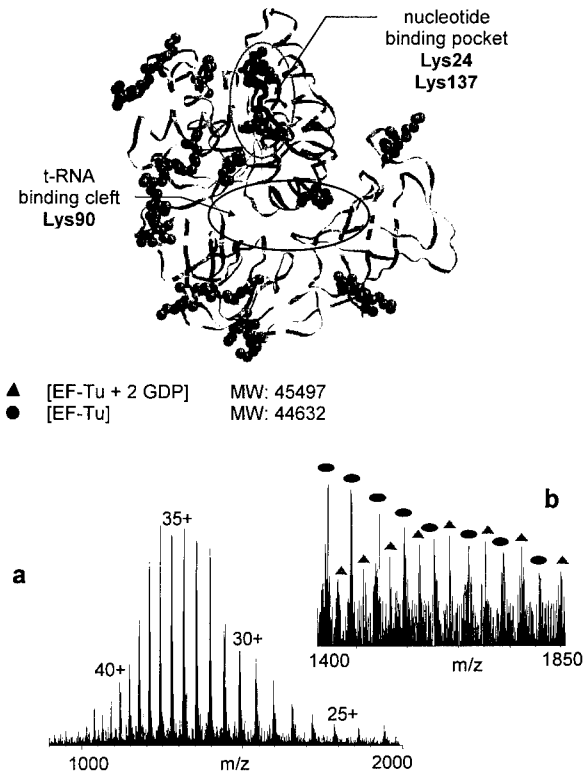


Fig. 4. ESI mass spectra and structure model of (a) free EF-Tu; (b) EF-Tu complexed with GDP. The residue numbers denote lysine residues found shielded in the EF-Tu/GDP complex towards acetylation [26].

ployed. Likewise, the molecular stoichiometry could be directly determined by ESI spectra of complexes prepared with di- and trinucleotide co-factors.

3. Conclusions and perspectives

Successful analyses by ESI-MS have been obtained recently for a variety of non-covalent supramolecular complexes of biomacromolecules and different types of binding, as demonstrated by coiled coil leucine zipper polypeptides and double-stranded oligonucleotides. This unexpected feature of the ESI method appears to open exciting perspectives for the molecular characterization of biomacromolecular interaction, with the consideration that little is still known about ion formation mechanisms and the effect of different solution structures on multiple charged ions in the gas phase. Further model studies on the chemical preconditions for formation and ESI-MS analysis of polypeptide-nucleotide complexes should lead to rapid improvements and are carried out at present in our laboratory. A key feature will be the investigation of high mass (low charge state) ions of complexes using suitable mass spectrometric instrumentation [26]. Applications to be anticipated include the use of ESI-MS as a tool for studying transcription (or translation) processes, receptor interactions, and the specificity of polynucleotide recognition at a molecular level. Furthermore, the direct analysis by ESI-MS is expected to be a useful complement for the characterization of protein-nucleotide interactions using chemical modification procedures.

4. Experimental procedures

Synthetic leucine zipper peptides employed in this study were prepared on an Applied Biosystems-430A, or a semi-automated ABIMED synthesizer, using Fmoc protection strategy as described previously [21]. Final purification of peptides was achieved by semi-preparative C_8-reversed phase HPLC. Oligodeoxynucleotides were synthesized on a Biosearch Cyclone DNA synthesizer using phosphoramidite chemistry and purified by C_{18}-HPLC before removal of the 5′-protecting group. Hybridization experiments were carried out in 1 M ammonium acetate by annealing at 10°C/h cooling from 80 to 20°C. Final desalting was performed on a Sephadex-G25 column with 10 mM ammonium acetate. ESI-MS was performed with a Vestec-201A quadrupole mass spectrometer (Vestec, Houston, TX) equipped with a 'thermally-assisted' electrospray interface [27]. The ion-spray interface temperature was approximately 40°C for all measurements. The mass analyzer with a nominal m/z range of 2000 was operated at unit resolution. An electrospray voltage at the tip of the stainless steel capillary needle of 2–2.2 kV and nozzle-repeller voltage difference of typically 10–50 V were employed. Mass calibration was performed with the 8+,

9 + and 10 + charged ions of hen egg white lysozyme and raw data analyzed by a Tecnivent vector-2 data system. High resolution ESI-MS measurements were performed with a Jeol JMS-102X double focusing sector instrument equipped with an Analytica electrospray interface (Analytica, Branford, MA) at a mass resolution of ~7000.

Peptide solutions (10–100 μM) were prepared in 2 mM ammonium acetate:methanol (9:1), and the pH adjusted with acetic acid and aqueous ammonia. Sample delivery was carried out by infusion through a 50-μm fused silica capillary at a flow rate of 4 μl/min using a Harvard-44 infusion pump.

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Multidimensional NMR spectroscopy of DNA-binding proteins: structure and function of a transcription factor

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Abstract

The solution structure of a type II DNA-binding protein (DBPII), transcription factor 1 (TF1), has been determined using NMR spectroscopy. A multidimensional, heteronuclear strategy was employed to overcome assignment ambiguities due to resonance overlap and broadened crosspeaks. This approach involved the use of selectively deuteriated, ^{13}C- and ^{15}N-labeled samples and 'isotopic heterodimers' to distinguish between intra- and intermonomeric NOEs. A comparison with the crystal structure and NMR analysis of the *E. coli* HU protein suggests that other homologous proteins in this family will possess similar tertiary structures. This NMR strategy is applicable to the study of other proteins and their biomolecular complexes.

Keywords: NMR spectroscopy; DNA-binding proteins; Structure determination; Transcription factor 1

1. Introduction

The interaction between proteins and nucleic acids accounts for many of the most important cellular functions such as the induction and/or repression of gene expression, assisted binding of other macromolecules to nucleic acids, and the packaging of nucleic acids into other superstructures. However, when damage occurs to one of the interacting molecules, disruption of normal cellular processes can occur. DNA, in particular, is susceptible to modification by endogenous and external environmental agents. Elucidating how these changes alter DNA structure and how specific repair proteins and enzymes recognize and bind these damaged, unusual bases is important for understanding how biological systems cope with their environment. A technique that is particularly well-suited for these types of studies is nuclear magnetic resonance (NMR) spectroscopy. By using NMR spectroscopy, we can compare the structures of the DNA molecule before and after chemical modification, or, with the appropriate protein-DNA complexes, identify any differences in intermolecular contacts, which may yield insights into how DNA recognition is accomplished.

We have been investigating the structure of the transcription factor 1 (TF1), a 22-kDa DNA-binding protein encoded by *Bacillus subtilis* phage SPO1, by high-resolution ^{1}H NMR spectroscopy [1,2]. TF1 is a member of the family of

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basic, dimeric proteins known as the type II DNA-binding proteins (DBPII). A highly resolved structure is known for only one DBPII, the *B. stearothermophilus*-encoded HU protein (*Bst.*HU) determined crystallographically by White and colleagues [3]. Recently, a NMR study of *Bst.*HU has also been carried out [4]. The high sequence homology between the DBPIIs suggests that the structure of *Bst.*HU is probably very similar to the other DBPIIs. It has also been inferred from the crystal structure of *Bst.*HU that DNA binding is effected by two concave β-ribbon 'arms'. Of the known DBPII, only TF1 and the integration host factor (IHF) from *E. coli* exhibit sequence-specific DNA binding. This has been explained by the presence of short extensions at the carboxy-terminus in TF1 and IHF. TF1 is unusual among the DBPIIs in that it binds preferentially to DNA containing 5-hydroxymethyluracil (hmU) in place of thymine [5,6]. TF1 has also been shown to bend DNA, and is capable of bending a 35 basepair DNA duplex through approximately 180° [7]. Although hmU can be a product of oxidative damage, the function of TF1 is presumably to inhibit transcription of SPO1 DNA (a double stranded 140-kbp genome in which all thymines are replaced with hmU) by RNA polymerase and is not involved in any DNA repair processes. However, by studying and comparing complexes of TF1 with hmU- and thymine-containing DNAs, TF1 may serve as a structural paradigm for understanding how proteins recognize unusual, cancer-related, or environmentally-damaged nucleotides.

NMR spectroscopy is unique in its ability to enable researchers to study biological molecules in solution with atomic resolution, but its applicability is limited in terms of the size of the molecule or complex that can be studied. The reasons for this limitation are increased line broadening, spectral overlap, and spin-diffusion effects [8]. These factors contribute to yield more complex spectra that are harder to interpret. From a spectroscopic standpoint, even though it is a homodimer, TF1 is a fairly large protein for ^{1}H NMR studies. The approaches used to overcome the problems associated with increased size have typically been to employ ^{13}C- and/or ^{15}N-labeling of the molecule in conjunction with multidimensional heteronuclear NMR experiments. While these methods have proven highly successful [9,10], we decided to develop a strategy involving selective ^{2}H-labeling of TF1. There are several compelling reasons for doing so, not the least of which are simplified and better-resolved spectra for resonance assignments, a means for extracting more accurate interproton distances, and the ability to study selected regions of the protein. These benefits have been discussed in detail previously [2,11–13].

We have also employed ^{13}C- and ^{15}N-labeled TF1 samples to verify the resonance assignments and to extend the number of unambiguously assigned intermonomer interactions in the homodimer. This concerted approach involving ^{2}H-, ^{13}C- and ^{15}N-labeling has been helpful not only for studying the free protein, but should also find useful application in the study of the protein-DNA complexes. Preliminary NMR studies have also been initiated in which the structures of hmU-containing DNA oligomers [14] and the corresponding thymine-containing oligomers will be compared for insights into possible structural aspects for nucleotide recognition. This type of comparison study is applicable to study the effects of any type of DNA modification, such as alkylation, deamination, photodimerization, or adduct formation.

2. Materials and methods

2.1. Production of selectively ^{2}H-labeled TF1

The overproducing plasmid pTF1X was used to produce TF1 in either *E. coli* MG1655 or the auxotroph DL39 [1]. A series of variants were produced by expressing TF1 in a media containing a mixture of uniformly ^{2}H-labeled amino acids and a 10-fold excess of selected unlabeled amino acids. For example, if the unlabeled amino acids were phenylalanine, glycine, isoleucine, and

tyrosine, and the remaining amino acids were ^{2}H-labeled, the variant protein was denoted as FGIY. A total of eight variants – FGIY, FVSY, LAS, VIFY, TE, PRAG, KMP, and Phe (the sole protiated residue was 2,4-[$^{2}H_2$]phenylalanine) – were produced. The deuteriated algal hydrolysates (isotopic enrichment 98%) were purchased from MSD Isotopes (Montreal, Canada); 1 g hydrolysate corresponded to approximately 0.5 g free amino acids, the remainder being inorganic salts and ^{2}H-labeled glucose. The bacteria were grown in 6 l of Luria broth until the cell density reached $A_{600\ nm} = 1$. The bacteria were spun down, resuspended in 1.0 l M9 minimal media with ampicillin, recentrifuged, resuspended in 1.5 l M9 media with ampicillin and incubated at 31°C for 15 min. Deuteriated algal hydrolysate and a 10-fold excess of the unlabeled amino acids were added to 1 g/l culture. Induction of TF1 synthesis was carried out at 41°C for 90 min. Procedures for the purification of TF1 have been described previously [15].

Uniformly ^{15}N- and ^{13}C-labeled TF1 samples were produced in a similar manner. ^{15}N- and ^{13}C-labeled algal hydrolysate were purchased from Cambridge Isotope Laboratories (Cambridge, MA). An 'isotopic heterodimer' composed of one normal TF1 monomer and one ^{13}C-labeled TF1 monomer was prepared by mixing equimolar amounts of normal TF1 and ^{13}C-labeled TF1 and allowed to equilibrate for 1 day at room temperature. 'Isotopic heterodimers' composed of different combinations of the selectively deuteriated variants were prepared in a similar manner.

2.2. NMR sample preparation

After purification, protein samples were exchanged into 400 mM NaCl, 100 mM phosphate, pH 6.8 buffer through repeated filtration using 10 000 molecular weight cutoff ultrafiltration units (Amicon, Inc.). Some samples were lyophilized and redissolved in 99.96% D_2O, others were dissolved in 90:10 H_2O/D_2O (%). Final protein concentrations were determined by UV absorbance measurements at 280 nm.

2.3. NMR spectroscopy

All NMR spectra were acquired on a Bruker AMX500 spectrometer equipped with an X32 Aspect 3000 computer. Two-dimensional experiments were collected with samples dissolved in 90:10 H_2O/D_2O (%) and some homonuclear experiments were collected with the samples dissolved in 99.96% D_2O. Three-dimensional experiments were collected with samples dissolved in 95:5 H_2O/D_2O (%). Except for the heteronuclear multiple quantum coherence (HMQC) experiment, the intensity of the solvent resonance was minimized by the application of a low power presaturating pulse during the recycle delay. Further suppression of the solvent signal was achieved by the incorporation of a jump-return sequence into the nuclear Overhauser effect spectroscopy (NOESY) experiments, a low power saturation pulse during the NOESY mixing time, and two high power trim pulses before and after the MLEV-17 sequence in the total correlation spectroscopy (TOCSY) experiment. Water suppression in the HMQC experiment was achieved by a jump-return sequence. Phase cycling enabled quadrature detection in t_1 and employed time-proportional phase incrementation (TPPI) in the absorption mode. All spectra were collected at 30°C.

Amino acid spin system identification and the subsequent complete sequential assignment of TF1 was accomplished through the concerted use of two-dimensional TOCSY, NOESY, ^{1}H-^{15}N HMQC, ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC), ^{1}H-^{13}C double half-filtered NOESY experiments, and three-dimensional TOCSY-NOESY and ^{1}H-^{15}N NOESY-HMQC experiments. The TOCSY experiment was collected with a spin-lock field of 12 kHz and a duration of 40 ms, and 35 ms in the three-dimensional experiment. NOESY spectra were collected with mixing times of 75, 100, 150 and 200 ms. The homonuclear three-dimensional TOCSY-NOESY and heteronuclear ^{1}H-^{15}N NOESY-HMQC experiments both employed NOESY mixing times of 100 ms. Two-dimensional ^{1}H-^{13}C double half-filter NOESY experiments

[16] were used to analyze the $^{1}H/^{13}C$ 'isotopic heterodimer'.

NMR data were analyzed using FELIX software (Biosym Technologies, Inc.) running on SGI Indigo and Indigo^{2} workstations.

2.4. Structure calculations

The structure determination was accomplished using a simulated annealing protocol with the XPLOR program [17]. For these calculations, NOESY crosspeak intensities were categorized into five corresponding distance intervals. Although the effects of spin diffusion are greatly attenuated in deuteriated proteins, crosspeak intensities in a series of NOESY data collected with different mixing times were evaluated for contributions due to spin diffusion. Pseudoatoms were used for methyl and nonstereospecifically resolved methylene groups and interproton distances were adjusted accordingly.

The first part of the calculations involved some all-atom Powell conjugate gradient energy minimization, followed by 6 ps of molecular dynamics at 1000 K, during which the force constants corresponding to molecular geometry were increased. This was followed by a slow cooling of the molecule to 100 K in 50-degree steps with 167 fs of dynamics at each temperature. Energy constants for repulsive terms were increased at each temperature to prevent further changes in the global conformation. The final part of the calculation involved another Powell energy minimization.

3. Results

3.1. Protein deuteriation and sample conditions

Perhaps the major limitation to the general application of NMR spectroscopy to the elucidation of biomolecular solution structures is directly related to molecular size. The ^{1}H NMR spectra of large biomolecules or complexes are characterized by severe line broadening due to slower tumbling rates and crosspeak degeneracies, both of which contribute to making assignment of individual crosspeaks problematic. Scalar-coupled (correlated spectroscopy (COSY), TOCSY, etc.) spectra suffer from a reduction in sensitivity due to larger line widths associated with increased molecular weight. NOESY spectra are made more difficult to analyze due to the increased number of protons, their availability for cross-relaxation, and the occurrence of spin diffusion effects which can lead to the appearance of false NOEs.

In some cases it is possible to dissect the protein of interest into smaller functional domains which can then be studied individually, otherwise there is little that can be done to physically reduce the size of a molecule. However, one method that has been shown to offset some of the detrimental effects of molecular size mentioned above is protein deuteriation. Because deuterium signals are not observed in high-resolution spectra and do not contribute to proton relaxation, deuteriation has two primary benefits – spectral complexity is reduced and sensitivity is increased (fewer relaxation pathways). Previous studies employed random fractional deuteriation in which the recombinant protein is expressed in the presence of deuteriated growth media, resulting in protein samples characterized by uniform levels of deuteriation. Our approach utilized samples with selected protiated amino acid types. The resulting proton spectra collected with these deuteriated variants exhibited improved sensitivity and were dramatically simplified (Fig. 1). The resonances of greatest intensity are associated with the protiated residues. Without the same number of proximal protiated residues, intraresidue dipolar couplings dominated the relaxation, while contributions from interresidue pathways were negligible, except those that were specifically chosen, as described below. As expected, relaxation measurements showed that selective deuteriation increased the longitudinal (T_1) relaxation times of the protiated residues [11].

By judiciously selecting the protiated amino acid types based on the known primary sequence of TF1 we were able to specifically assign short peptide segments throughout the protein using

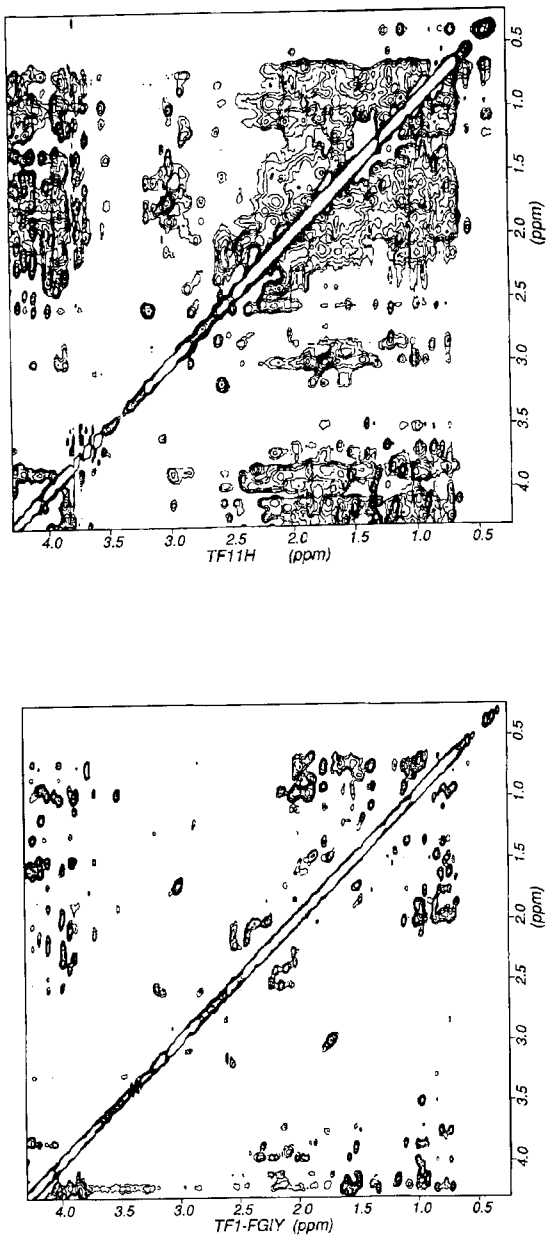


Fig. 1. (a) Aliphatic region of a NOESY spectrum of a fully protiated TF1 sample. (b) Corresponding spectrum of the FGIY variant.

the standard assignment procedures [18]. Through the concerted use of six of the deuteriated variants (the VIFY and Phe variants were only used for differentiating between inter- and intramonomeric contacts), we were able to specifically assign most of the TF1 resonances. The remaining ambiguous assignments were assigned and verified through the use of a uniformly ^{15}N-labeled TF1 sample [1].

However, before an NMR analysis of a protein can be conducted, sample conditions in which the protein exhibits biological activity and yields useful NMR spectra must be determined. At the sample concentrations normally used in NMR spectroscopy, some proteins exhibit a tendency to associate. This can lead to a large aggregate, causing line broadening. Sometimes aggregation can be minimized by varying sample conditions, in particular the ionic strength of the solution. While exploring the chaotropic series of salts is often beneficial, in the case of TF1 and we were able to reduce aggregation with higher concentrations of NaCl. The extent of aggregation is readily monitored via two-dimensional ^{1}H-^{15}N HMQC experiments. This experiment correlates the amide nitrogen with its attached proton. In the regime where aggregation is a factor, the nitrogen-proton correlations are typically broadened due to slower tumbling, sometimes broadened to the point that they are not observed in the spectra (Fig. 2a). At ionic strengths where association is disfavored, the peaks are sharper, and more abundant (Fig. 2b–c). We found that conditions of 400 mM NaCl yielded the best spectra, and just as importantly, represented conditions in which TF1 was still biologically active.

3.2. Determination of secondary structure

After a sequence-specific assignment has been completed, it is possible to determine the regions and types of secondary structure that are present in the protein. Advantage can be taken of the fact that the amide protons are easily exchanged with the bulk solvent (H_2O or D_2O) when present in flexible regions of the protein's tertiary structure, and more difficult to exchange when involved in hydrogen bonds in rigid secondary structure elements such as α-helices and β-sheets. Because of the short time that is required to collect ^{1}H-^{15}N HMQC data, this heteronuclear experiment is particularly well

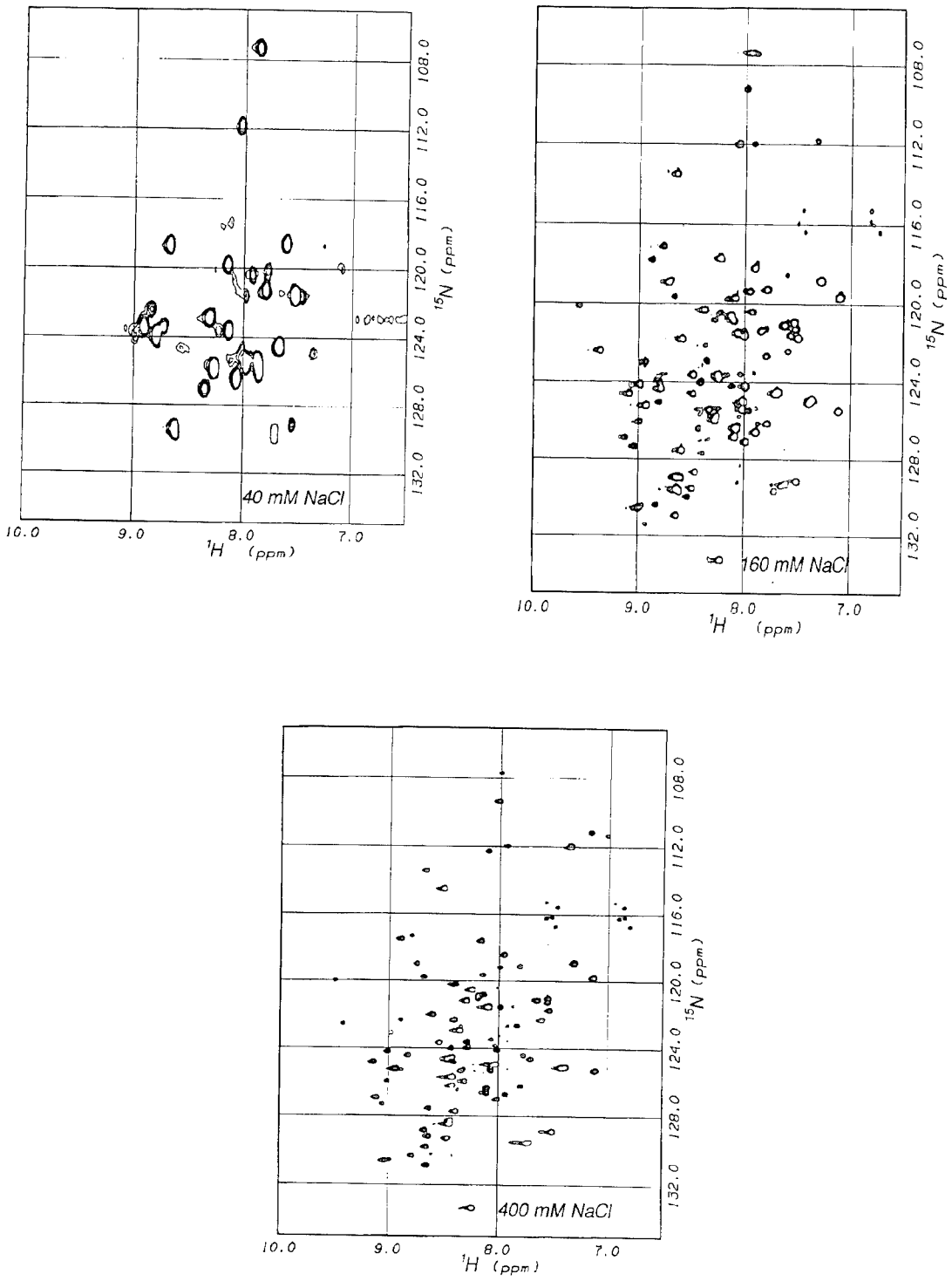


Fig. 2. Salt titration of TF1 as monitored by ^1H-^{15}N HMQC experiments. Each peak corresponds to an amide nitrogen-amide proton correlation. (a) 40 mM NaCl. (b) 160 mM NaCl. (c) 400 mM NaCl.

suited for these types of amide exchange studies. A uniformly ^{15}N-labeled TF1 sample was dissolved in H_2O, a ^{1}H-^{15}N HMQC dataset was acquired (Fig. 3a), and then the sample was lyophilized. The sample was then redissolved in D_2O and the exchange of the amide protons with the solvent deuterons was monitored over time. As can be seen in Fig. 3b–d, some amide protons resist exchange, even after 5 days. These protons correspond to residues involved in well-defined α-helices within the core of the protein.

Once the resonance assignments have been completed, it is then possible to tabulate all of the observed NOE (through-space) interactions. Certain patterns of NOEs are indicative of secondary structure. In particular, NOEs between an α proton and the amide proton three residues away (towards the C-terminal), and between an amide proton and the amide proton two residues away are representative of α-helices. A tabulation of observed NOEs is shown in Fig. 4. Without exception, those NOEs characteristic of α-helices also correspond to the most slowly exchanging amide protons (indicated by asterisks).

It has also been shown that the chemical shift of the α proton is indicative of secondary structure [19]. Specifically, when compared to the expected chemical shift for a corresponding residue in a unstructured, random configuration, the α proton in an α-helical residue is typically shifted upfield, whereas it is shifted downfield when present in a β-strand. In TF1, these chemical shift differences (Fig. 5) correspond well with where α-helices are expected from NOE and amide exchange studies, and where β-sheets are expected from NOEs and modeling studies. Perturbations suggest the presence of turns or kinks.

3.3. Intermonomer versus intramonomer NOEs

In terms of resonance assignments, the twofold symmetry of a homodimer is advantageous in yielding only half the spectral complexity of a heterodimer, but an issue that is extremely problematic and inherent with homodimers is the inability to distinguish between inter- and intramonomer NOEs. However, because TF1 establishes a monomer-dimer equilibrium in the absence of an appropriate DNA molecule [20], we were able to form 'isotopic heterodimers' by mixing equimolar amounts of different selectively deuteriated variants. Each sample was a mixture of two homodimers and the 'heterodimer' in a 1:1:2 molar ratio. Based on our hypothetical TF1 structure modeled after the crystallized *Bst*.HU structure, we identified interactions which we believed to lie at or near the monomer-monomer interface. If, for example, an NOE was observed between an alanine residue and a leucine residue in the NOE spectrum of the LAS sample, it would not be clear if the observed interaction was inter- or intramonomeric in nature. By forming the Phe-LAS 'heterodimer', this differentiation could be made. If the interaction were intermonomeric, the NOESY spectrum of the 'heterodimer' would show an NOE that was one-fourth the intensity of the NOE seen with the LAS sample. If the interaction was intramonomeric, the observed NOE would be half the intensity of the NOE seen in the LAS sample. In this manner we were able to unambiguously identify several inter- and intramonomer contacts.

A more elegant, straightforward and informative approach is to use heteronuclear half-filter NMR experiments. These pulse sequences allow the selection of protons according to whether they are covalently bound to NMR-active nuclei (in particular ^{13}C) or not [21,22]. In order to take advantage of these methods, equimolar amounts of an unlabeled sample were mixed with a uniformly ^{13}C-labeled sample, yielding a mixture of doubly-unlabeled TF1 dimers, doubly ^{13}C-labeled dimers, and 'isotopic ($^{13}C/^{12}C$) heterodimers', also in a 1:1:2 ratio. A modified two-dimensional double half-filter NOESY experiment [16] leads to the acquisition of two datasets. Summing the two datasets resulted in a spectrum that contained all the NOEs as before, but subtracting the datasets resulted in a spectrum that contained only those NOEs which involved a ^{13}C-bound proton and a ^{12}C-bound proton – in other words, only intermonomer NOEs (Fig. 6). These NOE crosspeaks could only arise from the

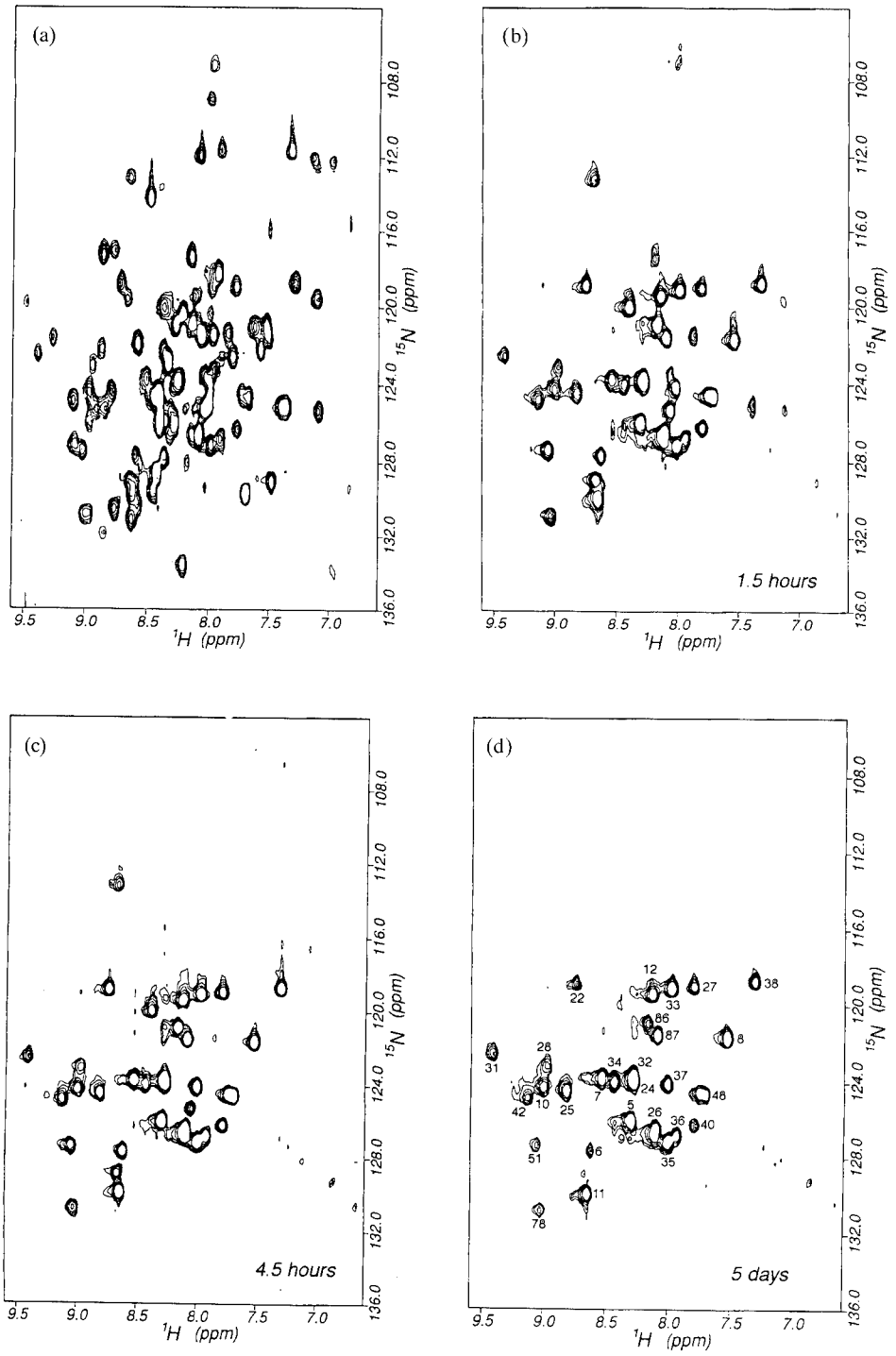


Fig. 3. Amide exchange rates as monitored by ^{1}H-^{15}N HMQC experiments. (a) In 90:10 H_2O/D_2O (%). (b) After 1.5 h in D_2O. (c) After 4.5 h in D_2O. (d) After 5 days in D_2O. Residue numbers corresponding to the observed amide correlations are indicated.

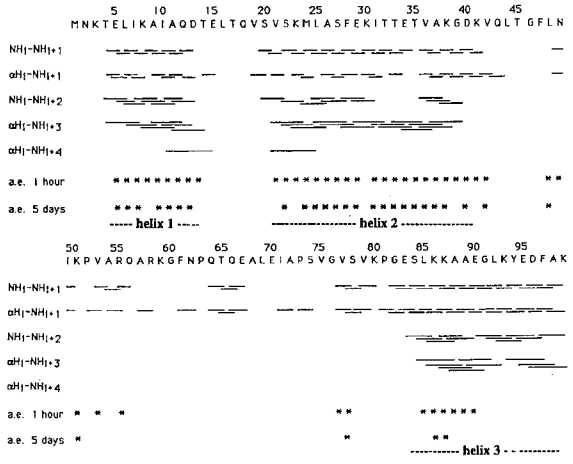


Fig. 4. A summary of sequential NOEs observed in TF1. Amide proton resonances that could be detected after exchange (a.e.) in D_2O for 1 h and for 5 days are marked with asterisks.

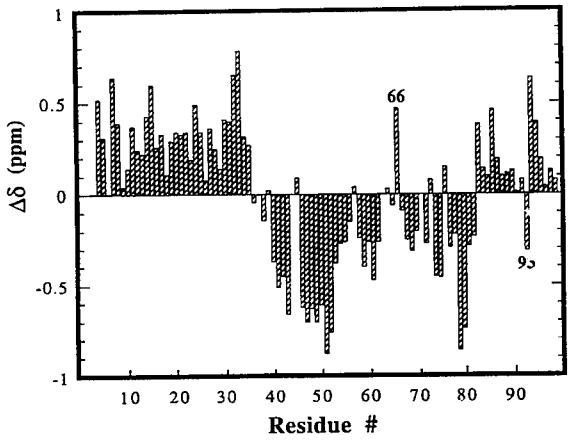


Fig. 5. Chemical shift analysis of α proton resonances of TF1. Chemical shift differences are calculated by subtracting the observed chemical shift from its 'random coil' value [19]. Positive chemical shift difference values indicate an upfield-shifted resonance.

'heterodimeric' species and not from either of the two 'isotopic homodimers'. From this experiment we were able to unambiguously identify 110 intermonomer NOEs.

3.4. Structure calculations

In addition to NOE information, we were also able to determine approximate backbone dihedral angles based on interresidue amide proton-α proton coupling constants. Altogether, 1599 intramonomer and 110 intermonomer interproton distance constraints and 208 dihedral angle constraints were used in our structure calculations. Ten calculations with starting structures based on the *Bst*.HU structure yielded a family of final structures (Fig. 7). Starting the calculations from extended structures also resulted in secondary structure elements – α-helices and β-strands – being located in the correct regions.

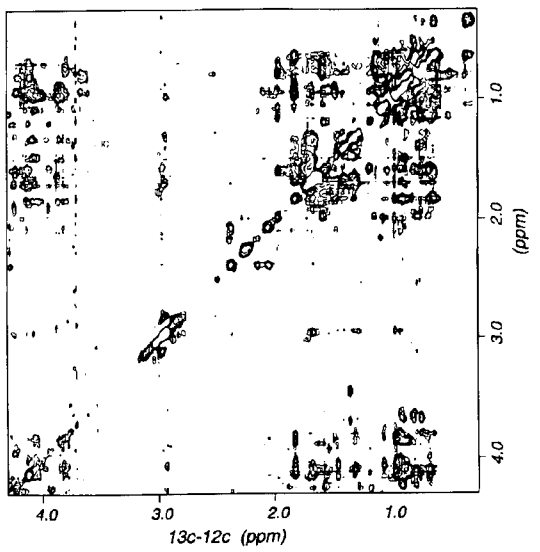


Fig. 6. Aliphatic region of a heteronuclear double half-filtered NOESY spectrum of a $^{13}C/^{12}C$ 'isotopic heterodimer' of TF1. These crosspeaks correspond to intermonomer interactions.

4. Discussion and conclusion

There are currently only two physical techniques that can be applied towards determining the structures of biomolecules and their complexes at atomic resolution – NMR spectroscopy and X-ray crystallography. Although a decided advantage of X-ray crystallography is the larger size of biological systems that can be studied, NMR spectroscopy is an extremely attractive technique since solution structures are determined, and often samples cannot be adequately crystallized,

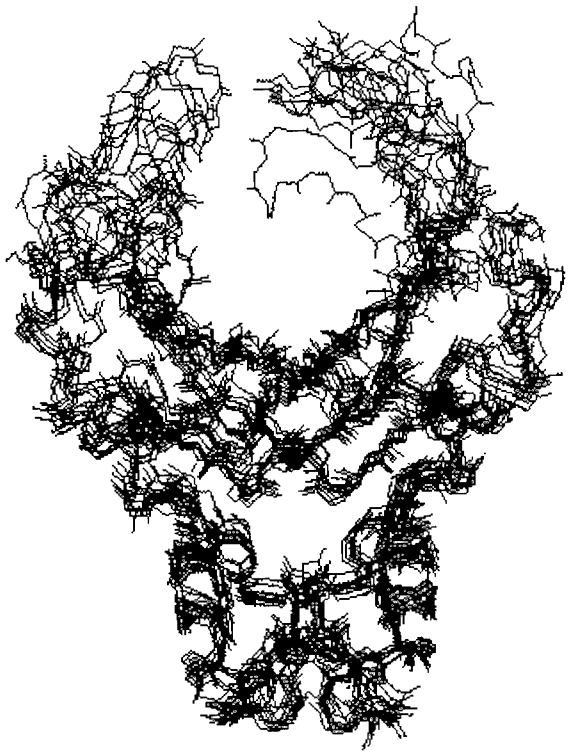


Fig. 7. Ten overlapping structures of TF1 from simulated annealing calculations based on NMR-derived distance and torsion angle constraints. Only backbone atoms are shown.

as has been the case with TF1. The ability to efficiently produce milligram quantities of isotopically enriched protein samples using molecular biology techniques combined with modern multidimensional NMR techniques has made it possible to study larger and larger molecular systems.

We have developed a strategy that employs selective deuteriation to enable the completion of resonance assignments for large proteins. Although ^{13}C- and/or ^{15}N-labeling of protein samples has been shown to be very useful and productive for NMR studies, deuterium labeling does offer some significant advantages. Three major benefits are: a dramatic simplification of the NMR spectra that greatly facilitates resonance assignments; a reduction in the number of potential relaxation pathways, resulting in improved resolution and sensitivity (compared to a fully protiated sample), as well as yielding more accurate interproton distance constraints (NOEs), which can then be used for improving structure determination calculations [11]; and the ability to use standard homonuclear NMR experiments for resonance assignments and NOE analyses. An additional benefit associated with selective deuteriation is that the residues and sequential regions that are protiated are easier to identify and can be studied in greater detail with fewer interfering resonances than the same regions in fully protiated or randomly deuteriated proteins. Because the primary sequence of the protein under study is usually known, these sequential regions may correspond to regions that are predicted to be structurally important or involved in biological activity based on other biochemical studies. That is, specific studies for identifying intermolecular contacts can be carried out using selective deuteriation even prior to completing a sequence-specific resonance assignment. The actual cost of producing selectively deuteriated variants is much lower than the prohibitive cost of producing selectively ^{13}C- or ^{15}N- labeled variants by adding selected fully ^{13}C- or ^{15}N-labeled amino acids during protein expression. A phenomenon to consider, however, before embarking on specific NMR studies involving deuteriation is biosynthesis. In the bacterial strain we used for overexpressing TF1, protiation occurred on the α carbon even for the mostly highly deuteriated variants. We were able to minimize the level of protiation without compromising overall protein yield by switching to an auxotrophic strain for expression. It should also be noted that the isotopically enriched algal hydrolysates do not contain equal amounts of the standard amino acids (asparagine, glutamine and tryptophan are particularly susceptible to degradation during hydrolysis). For example, TF1 is fairly lysine-rich with few aromatic amino acids compared to the hydrolysate, which leads to a differential level of deuteriation – phenylalanine and tyrosine residues in TF1 were almost fully deuteriated, whereas the deuteriation level for lysine residues was well below the mean level of 80% deuteriation.

The use of 'isotopic heterodimers' allowed us

not only to distinguish between inter- and intramonomer interactions, but also shows extreme promise for application to our studies of TF1-DNA complexes. Because we had a high-resolution structure of a homologous protein upon which to base our model of TF1, we were able to use 'isotopic heterodimers' of selectively deuteriated variants to verify certain expected intermonomer interactions. If this were not the case, we would have had to produce many more than eight variants to explore all possible interactions between the amino acid types. In terms of the costs and time required to generate and analyze all of the required selectively deuteriated 'heterodimers', the use of a $^{13}C/^{12}C$ 'heterodimer' would be extremely advantageous. Indeed, for distinguishing between inter- and intramonomer interactions in TF1, the use of such a 'heterodimer' proved to be an appealing solution because not only are all intermonomer interactions observed in the spectrum of one sample, but the need to quantitate relative NOE intensities was not required. In addition, analysis of the data from the double half-filter NOESY experiment yielded more intermonomer interactions than we obtained from the selectively deuteriated 'heterodimers'. Because of the larger number of intermonomer contacts, even in the double half-filtered NOESY spectrum there are still some overlapping crosspeaks due to resonance degeneracy. We are attempting to overcome this ambiguity by forming a TF1 'heterodimer' composed of a uniformly ^{15}N-labeled monomer and a uniformly $^{13}C,^{15}N$-double-labeled monomer for use with a three-dimensional heteronuclear half-filtered NOESY experiment in which the intermonomer NOESY crosspeaks can be further resolved according to amide nitrogen resonance frequencies.

The structure of TF1 that was determined using our NMR-derived constraints is very similar to our preliminary model based on the *Bst*.HU structure – a homodimeric protein with a core formed from two helices from each monomer, wrapped by two three-stranded β-sheets, each of which leads into the hypothesized DNA-binding β-ribbons. Other similarities include the fact that the most rapidly exchanging amide protons are in the DNA-binding β-ribbons, suggesting that these are the most structurally flexible domains in TF1, and were also the region with the highest R values in the *Bst*.HU crystal structure [3]. There are, however, differences between the calculated and model structures – the first helix is shorter by two residues, the second helix starts and ends earlier than in the crystal-based structure, the β-strands are slightly different, and the terminal nine amino acids in TF1 that are not present in *Bst*.HU extend the third α-helix to the C-terminus. A comparison of secondary structure elements between the crystal structure and the NMR analysis of *Bst*.HU [4] with our NMR analysis of TF1 is shown in Fig. 8. As can be seen, the structural agreement in these three analyses is very good, suggesting that other homologous DBPII will have the same general structure.

A calculation of potential charge distribution on the solvent-accessible surface of the NMR-derived TF1 structure presents a possible mechanism for the recognition of hmU nucleotides and for DNA bending. A patch of positive charge exists in a β-ribbon saddle formed from the extension of the two three-stranded β-sheets into the DNA-binding β-ribbons. Near the ends of these β-ribbons are the phenylalanine residues at position 61. In all other DBPII, an arginine residue is absolutely conserved at this position. Substitution of Phe61 with arginine abolishes

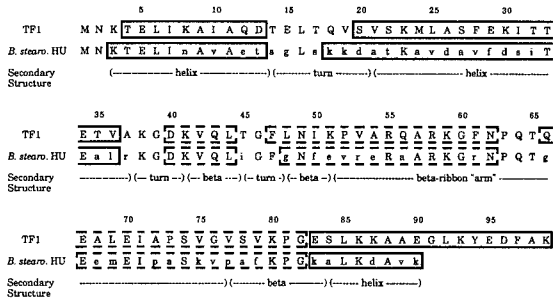


Fig. 8. A comparison of secondary structure analysis of TF1 and *Bst*.HU [4] by NMR spectroscopy. *Bst*.HU secondary structure elements as determined by X-ray crystallography [3] are indicated below the sequences. Conserved residues are identified by capital letter in the *Bst*.HU sequence. α-Helices are indicated by the solid boxes, β-sheets by dashed boxes.

preferential binding to hmU-containing DNA by TF1 [5]. Furthermore, deletion of the nine amino acids at the C-terminus drastically reduces TF1's ability to bind to either hmU- or thymine-containing DNAs [5]. We hypothesize that DNA binds to the positively charged saddle, leaving the β-ribbons and C-terminal helices spatially disposed in a manner that suggests that these two structural motifs are capable of bending and 'holding' the DNA across the face of TF1 on either side of the saddle with hmU recognition occurring via Phe61. We are currently investigating this possible mechanism by studying complexes of TF1 with DNA oligomers containing either hmU or thymine. In order to observe only intermolecular contacts, we are using a uniformly ^{13}C-labeled TF1 homodimer in conjunction with the unlabeled DNA oligomers (the cost of isotopically enriching DNA in NMR quantities is overwhelmingly prohibitive). In this case, a double half-filtered NOESY experiment will allow us to rapidly identify TF1 residues that are directly involved in contacts with the DNA. Employing the standard double half-filter pulse sequence [21,22] will also allow us to selectively observe either intra-TF1 or intra-DNA NOEs and thus evaluate any structural changes in either interacting molecule. These studies are underway.

We have demonstrated a strategy for structural NMR investigations involving a combination of selective deuteriation and heteronuclear half-filtered experiments. This approach should be generally applicable not only for the study of dimeric DNA-binding proteins, but also for studying other larger proteins and their complexes with other molecules. Heteronuclear experiments can also yield a wealth of information concerning protein dynamics, which serves to indicate and underscore the continuing development, evolution and future directions for applications of biomolecular NMR spectroscopy.

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Structure and dynamics of the *lac* repressor-operator complex as determined by NMR

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Abstract

The structures of the *lac* repressor headpiece and of its complex with an 11 base-pair *lac* half-operator have been determined by NMR spectroscopy. By ^{15}N relaxation studies the dynamic behaviour of the free protein and of the protein in the complex could be established. In the three-helical *lac* headpiece local backbone mobility was detected in the N-terminal and C-terminal peptide regions and in the loop between helices II and III. Upon DNA binding this loop becomes more rigid and it changes its conformation considerably. The specificity of the protein-DNA interaction follows from a large number of hydrogen-bond and hydrophobic interactions between amino acid side chains and DNA backbone and bases. Restrained molecular dynamics calculations suggest that some of these interactions are dynamic in nature.

Keywords: NMR spectroscopy; Protein-DNA interaction; Flexibility and dynamics; *Lac* repressor; *Lac* operator

1. Introduction

The recognition of DNA sequences by proteins lies at the heart of many cellular processes, such as gene regulation, replication and DNA repair. Therefore, the mechanism of protein-DNA interaction constitutes a major problem in molecular biology. Approaches to this problem range from biochemical and genetic studies (mutagenesis of DNA-binding proteins) to the application of the methods of structural biology (X-ray crystallography and NMR spectroscopy). In the early 1980s, the first crystal structures became available for a number of bacterial proteins such as λ and cro repressors and CAP (for recent reviews see [1–3]). NMR spectroscopy started to contribute around 1985 with the structure elucidation of the *lac* repressor headpiece [4] and a low resolution structure of the headpiece-operator complex in 1987 [5]. While the first structures all contained a helix-turn-helix motif as the essential DNA-binding subdomain, later a plethora of DNA-binding motifs was characterized including zinc-fingers, leucine zippers, helix-loop-helix proteins and even β-sheet DNA-binding proteins. NMR has significantly contributed to this, since DNA-binding domains of proteins are often relatively small independently folded domains that can be expressed and studied separately. Thus, in particular for the various subclasses of zinc-fingers, the first structural information came from NMR. This was the

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case for the TFIIIA-type zinc fingers [6,7] for the nucleocapsid proteins [8] and for the nuclear hormone receptors [9,10]. Often a combination of various biochemical and genetic methods with structural work is most fruitful for understanding protein-DNA recognition on a molecular level. X-Ray or NMR structures of protein-DNA complexes allow an interpretation of the results of mutagenesis, which would be very difficult in the absence of structural data. Conversely, binding affinities of mutant proteins or DNA variants give insight in the importance of the various interactions seen in X-ray or NMR structures in terms of the free energy of binding.

The *lac* repressor is a case in point. For this system over the years an enormous body of biochemical and genetic data has been generated, which can now be interpreted in structural terms. A staggering number (thousands) of mutants has been characterized, mostly by the work of Miller [11] and Lehming et al. [12]. In particular, mutant proteins with altered DNA-binding specificity are informative in this respect. With recent structure refinement of the *lac* headpiece-operator complex by NMR, this work can be put on a firm structural footing [13]. In this chapter we shall discuss the *lac* repressor-operator system as an example to illustrate the NMR approach to protein-DNA recognition.

2. *Lac* repressor headpiece

The *lac* repressor of *E. coli* is a tetrameric protein of molecular weight 154 000. The native repressor is too large for high-resolution NMR studies. However, each subunit has a separate DNA-binding domain (headpiece) that can be cleaved off by proteolytic enzymes [14]. The amino acid sequence of the N-terminal region is shown in Fig. 1. Depending on the proteolytic enzyme used, headpieces can be prepared containing 51, 56 or 59 amino acid residues (HP 51, HP 56 or HP 59). These headpieces retain their original three-dimensional structure and their ability to recognize the *lac* operator specifically [15]. The trypsin-resistant core is involved in the subunit interaction and contains the inducer

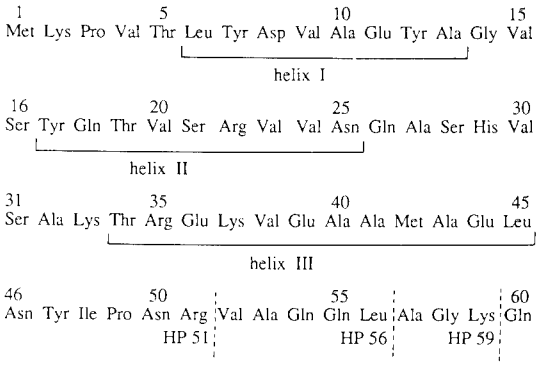


Fig. 1. Amino acid sequence of the N-terminal part of the *lac* repressor. The α-helical regions as determined by NMR are indicated. Cleavage sites are shown of the enzymes clostripain, chymotrypsin and trypsin, yielding headpiece fragments of 51, 56 and 59 amino acid residues, respectively.

binding site. The sequence of the natural *lac* operator reveals an approxiate twofold symmetry [16] and in agreement with that, two subunits of the *lac* repressor suffice to recognize the *lac* operator [17]. Therefore the *lac* repressor should bind with two headpieces to each half of the operator. The *lac* headpiece (HP 51) was one of the first proteins for which the three-dimensional structure was determined by NMR [4,18]. Recently, the structure has been refined using a more extensive set of constraints from NOEs and J-couplings [19]. The family of structures is shown in Fig. 2. *Lac* headpiece consists of three helices, the first two of which constituting the helix-turn-helix motif, while the third packs against this subdomain forming a hydrophobic core. It can be clearly seen that the loop between helix II and III shows a larger conformational variability than the helical core of the protein. From ^{15}N relaxation data measured for bacterially expressed HP 56 it is clear that this corresponds to a larger backbone flexibility for the loop region. As is discussed below this flexibility is functional as it allows the protein to adjust to the DNA upon complex formation.

3. *Lac* headpiece-operator complex

Lac operator of *E. coli* is defined genetically as the control region in the *lac* operon, where

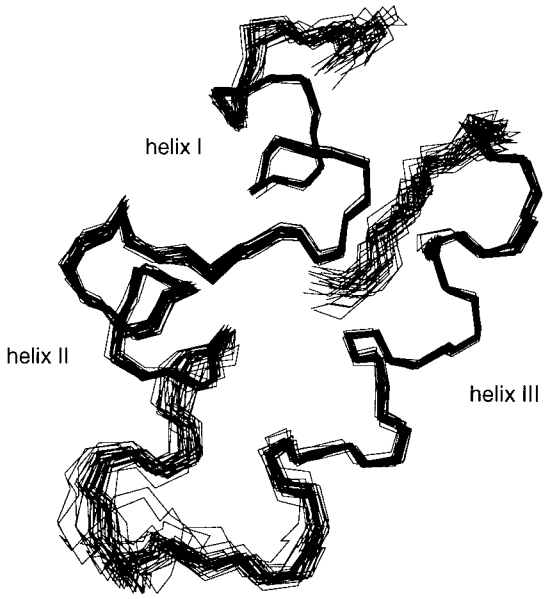


Fig. 2. Superposition of 30 structures for the *lac* headpiece (residues 3–48). The three α-helices are indicated. The structures were calculated using distance geometry and restrained Molecular Dynamics procedures based on ca. 950 distance constraints from NOEs and 46 dihedral angle constraints [19].

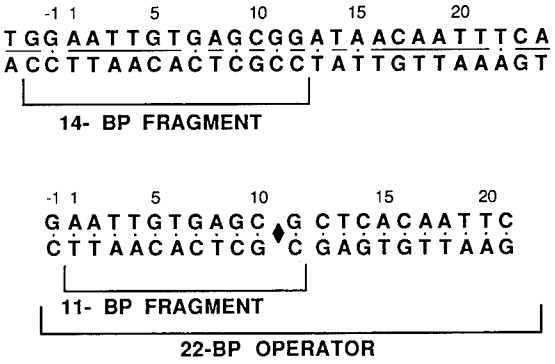


Fig. 3. Sequences of native *lac* operator (a) [16] and 'ideal' symmetric *lac* operator (b) [20,21]. Synthetic operator fragments of 11, 14 and 22 bp used in the NMR studies are indicated.

operator constitutive mutants occur. The region protected by the *lac* repressor is 20–25 bp long, with a pseudo-dyad axis going through GC 11 [16]. It was found later that symmetrical *lac* operators lacking the central GC base-pair bind *lac* repressor up to an order of magnitude stronger than the native one [20,21]. The sequences of the operators and the fragments discussed here are shown in Fig. 3. The initial NMR studies were made with the 14-bp fragment, which turned out to be the stronger binding half-operator as it also occurs in the symmetrical operator (Fig. 3b). The binding affinity of the isolated headpiece with a half-operator is not extremely high ($K_d \approx 10^{-6}$ M), so that the free and DNA-bound forms are in fast exchange on the NMR time-scale. This greatly helps in assigning the resonances in the NMR spectra of the protein-DNA complex, since the resonances of the free species can be followed in titrations, either by adding, for instance, protein to DNA or by titrating the complex with increasing amounts of salt, by which it will gradually dissociate. Fig. 4 shows one of the most readily accessible regions of a 2D NOE spectrum of the complex of HP 56 with the 14-bp operator. It contains a window, where only intra-DNA cross-peaks occur (H6/H8-H1′ and cytosine H5-H6). These cross-peaks provided a start for the assignment of the DNA resonances, as is shown in Fig. 4 for one strand by the lines connecting intra- and internucleotide cross-peaks. In this way, assignments were obtained for all non-exchangeable protons of the DNA in the complex, except for some of the H5′ and H5″ protons. The general pattern of intra-DNA NOEs is still that of a B-DNA type conformation. Also, most of the ^{1}H resonance positions show small shifts upon complex formation, with a maximum of 0.2 ppm for the H8 proton of G5 and the H1′ proton of G7. These results are also consistent with the idea that small adjustments of the DNA conformation occur. Similarly, a large number of ^{1}H assignments have been made for the protein part of the HP 56-14 bp operator complex. For this, a combination of 2D NOE spectra and homonuclear Hartman–Hahn (HOHAHA) spectra was used.

The early low-resolution structure of the HP 56-14 bp operator complex was obtained by docking the protein to DNA in standard B conformation guided by 11 NOEs observed between protein and DNA [5]. This model was similar to other complexes of helix-turn-helix

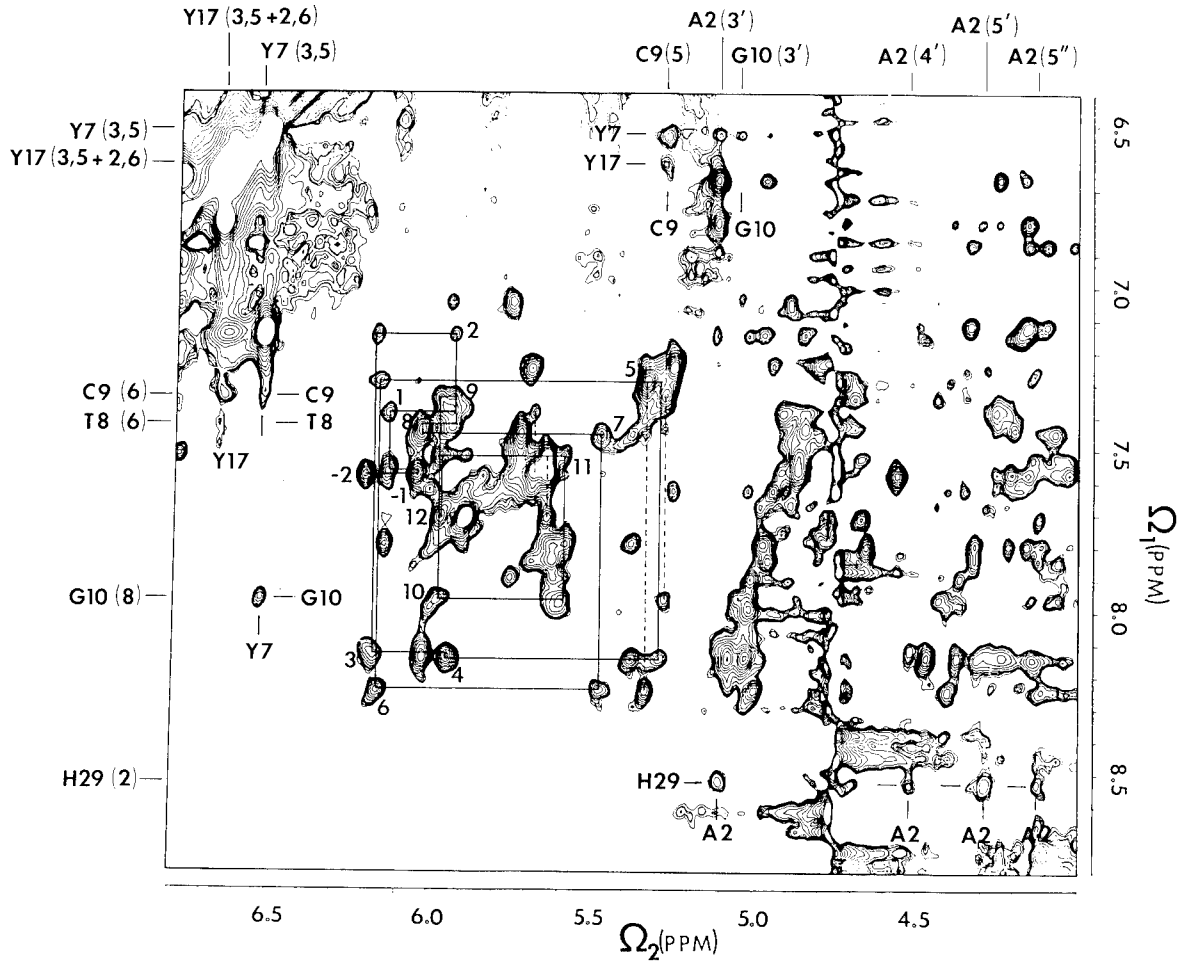


Fig. 4. Part of the 500-MHz 2D NOE spectrum of the HP 56-14 bp operator complex. Sequential NOEs of one of the DNA strands are indicated by the connecting lines. The protein-DNA NOE cross-peaks present in this part of the spectrum are also indicated.

proteins in that helix II of the headpiece, the 'recognition helix' of the helix-turn-helix motif, is inserted in the major groove of DNA and makes the majority of the protein-DNA interactions. However, a surprising result was that the orientation of the recognition helix was opposite to that found in all other known complexes. This means that the first helix points towards the dyad axis of the operator, while for other proteins, such as λ and cro repressors and CAP, it points away from it. Genetic experiments by Lehming et al. [12] confirmed this different orientation. These authors constructed a *lac* repressor mutant with the first two amino acids of the recognition helix replaced by those of *gal* repressor (Tyr 17 → Val, Gln 18 → Ala). This mutant repressor had high affinity for the *gal* operator, which differs from the *lac* operator at positions 7 and 9. Although this already gives some clue as to the orientation of the recognition helix, a more definitive result was their finding of a repressor mutant with Arg 22 replaced by Asn, which now had specificity for a *lac* operator with GC5 replaced by TA. This fixes unambiguously the orientation of the recognition helix as the opposite of that of cro and λ repressors.

Studies of a complex of two HP 56 molecules with a symmetric 22-bp *lac* operator [22] showed that the two headpieces bind independently and in essentially the same binding mode as they do in half-operator complexes. Therefore, a more detailed study was made of a complex with an 11-bp half-operator (Fig. 3b) [23]. Based on a larger number of NOEs, among which 39 between protein and DNA, a restrained molecular dynamics refinement of the headpiece operator complex was carried out [13]. Some statistics of the calculation are shown in Table 1. We note that the protein-DNA complex was put in a box containing over 3000 water molecules to which salt ions were added, and periodic boundery conditions were applied. Inclusion of solvent was felt necessary for a reliable simulation and also because water molecules may play an important role in the interface between protein and DNA. A fairly long equilibration of 60 ps was necessary primarily to equilibrate the ion distribution around the complex. Then a 85-ps trajectory was used for analysis. In addition an annealing procedure was undertaken in order to assess the precision of the structure determination. This consisted of heating the complex to 1000 K and letting it cool down to 300 K in a 5-ps RMD calculation followed by energy minimization. Six structures were obtained by this procedure using different snapshots from the trajectory as starting points.

The RMD runs yielded a satisfactory structure for the complex with a low total energy (-175×10^3 kJ mol^{-1}) while the restraint energy did not exceed the average thermal energy. There were on average 42 bounds (out of 980 total), which were violated by 0.5 Å or more, mostly within the DNA (none involving protein-DNA contacts).

Among a set of 15 structures, nine from the trajectory and six from the annealing procedure, the r.m.s.d. for backbone atoms for both DNA and protein (residues 4–48 for the headpiece) with respect to the mean was found to be 0.9 Å. This value can be considered as a measure of the precision of the structure determination. Fig. 5 shows the average backbone confirmation of the headpiece in the complex compared with that of the free protein. It is clear that the loop between helix II and helix III has undergone a considerable conformational change. In particular Asn 25 changes its ϕ/ψ angles such that the exit from helix II is quite different in the free and complexed protein.

From the analysis of the 85-ps trajectory a

Table 1
Restrained molecular dynamics refinement of the *lac* headpiece-11 bp operator complex

Start	Optimized vacuum structure
Size	989 atoms complex, 28 Na^+, 10 Cl^- 3346 waters
Constraints	Intra protein 260
	Intra DNA 241
	Protein-DNA 39
Forcefield	GROMOS
Trajectory	60-ps equilibration
	85-ps analysis
Annealing	(6×) 1000 K → 300 K ↑ [5 ps]

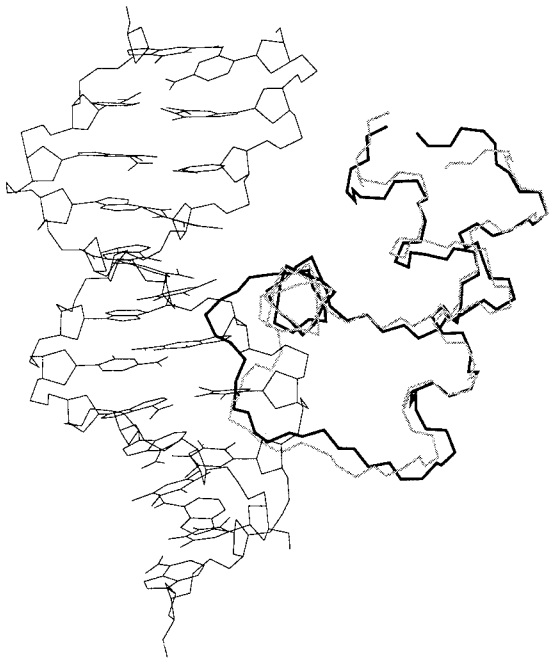


Fig. 5. Backbone trace of the *lac* repressor headpiece for the free protein (grey line) and the protein in complex with operator DNA (dark line).

picture emerged of the interactions by which the *lac* operator is recognized by the headpiece. Apart from electrostatic interactions, which are probably non-specific, a number of hydrogen bonds are observed between protein and DNA, both to the sugar-phosphate backbone and to the bases. In addition an extensive network of apolar interactions is observed involving the side-chains of Tyr 17, Gln 18 and Ser 21, and the methyl groups of Thy 6 and Thy 8. These interactions are schematically shown in Fig. 6. Two direct hydrogen bonds between amino acid side-chains and bases are seen, which appear to be essential for specific recognition. These are the ones between Gln 18 and Cyt 7 and between Arg 22 and Gua 5. Important anchoring hydrogen bonds with DNA phosphates include those of the amide NH of Leu 6 and the side-chain amide of Asn 25. These are conserved among the whole family of helix-turn-helix proteins. Histidine 29, present in the loop between helices II and III is involved in a multitude of interactions: electrostatic interactions because of its positive charge,

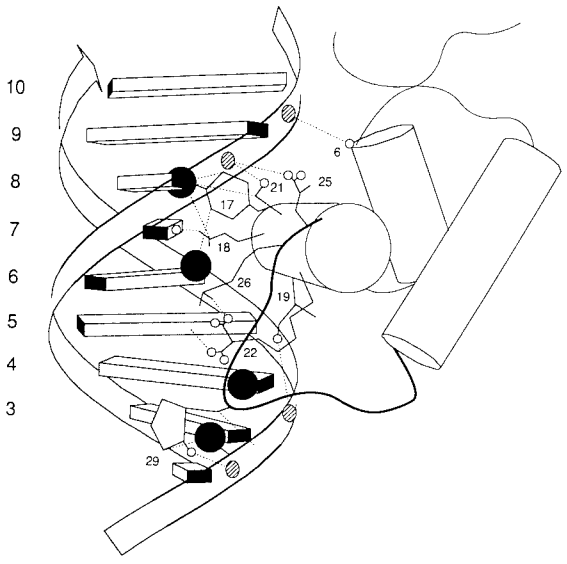


Fig. 6. Schematic view of the interactions between *lac* headpiece and *lac* operator. Shown are the hydrogen bonds between protein and DNA and the apolar interactions in the hydrophobic cluster formed by side-chains of Tyr 17, Ser 21, Gln 18 and the methyl groups of Thy 6 and Thy 8. Thymine methyl groups are shown as solid circles, phosphate groups are striped circles and protons in H-bonds as small circles.

a hydrogen bond with a phosphate group and van der Waals interactions with the methyl group and sugar ring of Thy 3. For a more complete description of these interactions we refer to the paper by Chuprina et al. [13].

4. Dynamics of free and complexed *lac* headpiece

Using ^{15}N-labeled HP 56, the backbone dynamics of the headpiece has been studied for the free protein and in the complex with the operator [19]. Fig. 7 shows ^{15}N-$T_{1\rho}$ data for the backbone amide nitrogens. The N-terminal and C-terminal region show a larger mobility than the core of the protein, both in the free and in the bound state, as evidenced by larger values of $T_{1\rho}$. Comparing the relaxation data of Fig. 7A and B it is clear that in general the $T_{1\rho}$ values in the complex are lower than in the free state due to the higher molecular weight. However, more significantly, the higher mobility in the loop region around residue 30 observed for the free protein disappears in the complex. This suggests that the flexible loop adapts itself to the DNA and becomes more rigid. This behaviour is consistent with a negative change in heat capacity upon complex formation, which was interpreted

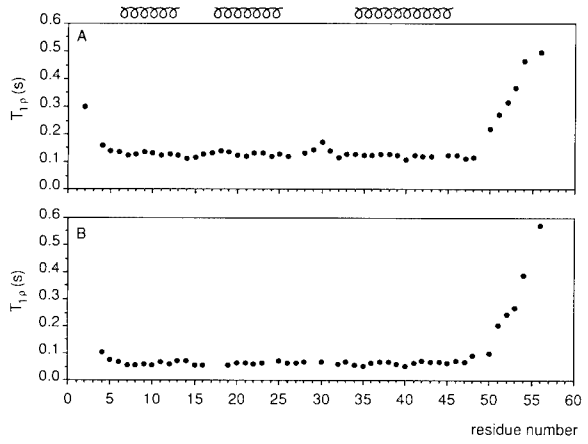


Fig. 7. ^{15}N-$T_{1\rho}$'s of the amide nitrogens vs. residue number for free HP 56 (A) and for HP 56 complexed to the *lac* operator (B). The three α-helices of the headpiece are indicated on top.

by Spolar and Record [24] as a (partial) folding transition in the repressor. Based on thermodynamic data this adaptability accompanied by partial folding has been suggested to be a more general phenomenon in protein-DNA recognition [24]. For the *lac* headpiece the present structural and relaxation data support this view.

Although part of the protein backbone adopts a more rigid conformation, there is also evidence for residual flexibility in the protein-DNA interface. This comes from an analysis of the restrained MD trajectory in terms of hydrogen-bonds between protein and DNA showing that some of these hydrogen-bonds are not rigid but fluctuate in time. Thus, while anchoring H-bonds involving Leu 6 and Asn 25 are formed close to 100% of the time, the one between Arg 22 and Gua 5, for instance, breaks up during the trajectory and is replaced by an intra-protein H-bond with Glu 26. Apparently, alternative hydrogen bonding schemes are possible with very similar energies. We believe that indeed protein-DNA complexes are rather dynamic in nature and that the current more rigid picture of these complexes needs correction.

5. Comparison with genetic data

Ebright [25] has demonstrated that wild-type *lac* repressor with Gln 18 substituted by Gly, Ser and Leu loses the ability to distinguish between the base pairs GC, TA and AT at position 7. In contrast, all three substituted variants are able to discriminate at position 5, 6, 8, 9, and 10. Consequently, Ebright proposed that Gln 18 contacts base pair 7. Based on the affinity of repressor variants to operators with particular base pair substitutions Müller-Hill and co-workers [26,27] suggested a direct contact between Arg 22 and base pair GC 5 in addition to the Gln 18-GC 7 contact. Both contacts are found in our structural model.

From the work of Caruthers [28], a hydrophobic interaction involving the methyl group of Thy 8 is known to be essential for binding of the *lac* repressor. We observe a stable interaction between this methyl group and a hydrophobic pocket formed on the protein by Tyr 17 and Ser 21. The carbonyl Cδ of Gln 18 is also close to the Thy 8 methyl. These results correlate nicely with the mutant studies by Sartorius et al. [29] indicating that residues in the fifth position of the recognition helix (Ser 21 in wild type) contribute to the recognition by residues 1 and 2 (Tyr 17 and Gln 18 in wild type). Although no firm evidence was found for a direct specific contact of Ser 21 to any base pair, the authors suggested that Ser 21 is involved in the recognition of bp 7 (our numbering). This is not corroborated by our results.

Sartorius et al. [27] concluded that the specific binding of *lac* repressor mutants with amino acid exchanges at positions 1 and 2 of the recognition helix is mainly directed towards bp 7 and to a lesser extent towards bp 6 of the *lac* operator (our numbering). This is not incompatible with our results, that show interactions between Gln 18 and bp 7, and between Tyr 17 and bp 6, 8 and 9. Interactions with bp 8 and 9 have not been observed in the genetic studies by Sartorius et al. [27,29], which is probably due to the fact that no repressor mutants were identified with a non-negligible affinity for any *lac* operator variant substituted in bp 8. Apparently Thy 8 is a crucial element in repressor-operator recognition. Furthermore, substitutions at positions 1 and 2 of the recognition helix apparently lead to rearrangements of side chains, giving rise to amino acid-base pair correlations in complexes of mutant operators with mutant repressors that are somewhat different from the correlations in the wild type complex.

In our structure, Asn 25 makes a hydrogen bond contact with the Thy 8 phosphate, as has been proposed by Sartorius et al. [29] based on genetic data. Thr 19 and Ser 21 are also important for repression, since their substitution almost invariably leads to an I^- mutant [30]. Our data suggest that the observed loss of repression upon replacement of these amino acids may be due to loss of the operator contacts formed by these residues. Thus Thr 19 appears to be important not only for the protein tertiary structure, as suggested by Boelens et al. [5], but also for DNA binding. Ser 21 is not only involved in a

base specific contact to Thy 8, as discussed above, but in a phosphate contact as well.

We can conclude that our structure of the *lac* headpiece-operator complex agrees very well with the genetic data and indeed provides a basis for a detailed interpretation of these data in structural terms.

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Genetic and environmental factors in the etiology of human brain tumors

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Abstract

Experimental studies in rodents using chemical carcinogens and viral oncogenes show a high susceptibility to malignant transformation. Analytical epidemiological studies have revealed an increased risk of human brain tumor development in association with certain occupations but, with the exception of therapeutic X-irradiation, attempts to identify a specific exposure or causative environmental agent have so far been unsuccessful. Thus, endogenous mutations and genetic factors may play a more important role. This view is supported by recent studies on the nature of DNA alterations in human brain tumors. More than 70% of *p53* mutations observed during glioma progression are G:C→A:T transitions, predominantly at CpG sites, i.e. likely to be produced by deamination of 5-meC or related spontaneous mechanisms. No specific mutations or mutational hot spots were found which could be suggestive of environmental carcinogens operative in the etiology of human brain tumors. A similar pattern of mutation is found in colon cancer, sarcomas, and lymphomas, i.e. neoplasms with largely unknown etiology. This is similarly true for *p53* germline mutations which again show a strong preference for G:C→A:T transitions at CpG sites.

Keywords: Neuro-oncogenesis; Nitroso compounds; Viral oncogenes; Retroviral vectors; Cancer epidemiology; Gliomas; *p53*; Germline mutations

1. Introduction

Although brain tumors amount to less than 2% of all malignant neoplasms and thus constitute a small fraction of the overall human cancer burden, their clinical significance is considerable. A significant proportion of central nervous system (CNS) neoplasms affects children in which tumors of the nervous system (including retinoblastomas and peripheral neuroblastomas) rank second in incidence after leukemias. In recent years, considerable progress has been made in the understanding of tumor evolution in the CNS, particularly in the areas of immunology and molecular genetics of gliomas. However, the etiology of brain tumors is, with the exception of familial cancer syndromes involving the nervous system, still largely unknown. In the following we will briefly review the current body of knowledge, in the fields of experimental animal studies, epidemiology and molecular genetics.

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2. Chemical neuro-oncogenesis in experimental animals

Several classes of chemical carcinogens have been shown to selectively induce tumors in the rodent nervous system. The most effective compounds are simple alkylating agents, in particularly nitrosourea-derivatives which cause CNS neoplasms in rats after systemic administration [1]. Dialkyl-aryltriazenes [2] and azo-, azoxy- and hydrazo compounds [3] are similarly effective. Ethylnitrosourea (ENU) and related ethylating agents are particularly powerful when administered as a single (pulse) dose transplacentally or shortly after birth [4]. With these agents, the susceptibility of the rat CNS begins at the 10th prenatal day (E10), increases gradually and reaches its maximum at birth when a single dose is approximately 50 times more effective than in adult rats. After birth, the susceptibility of the CNS decreases and reaches that of adult rats at approximately 1 month. *N*-Nitrosomethylurea and related methylating agents produce a high incidence of CNS neoplasms when given as multiple small, weekly doses to adult rats. Brain tumors induced by alkylating agents have been classified as oligodendrogliomas (originating from the white matter), astrocytomas and mixed gliomas (originating from the subependymal plate). ENU-induced neoplasms previously classified as ependymomas express the neuronal marker synaptophysin and may thus better be termed primitive neuroectodermal tumors with neuronal differentiation. In addition, transplacental and perinatal exposure causes the induction of a high incidence of malignant schwannomas of the cranial and peripheral nerves [4].

Malignant transformation by alkylating agents is thought to result from interaction of the ultimate carcinogen, i.e. a methyl or ethyl cation, with cellular DNA. The major promutagenic base is O^6-alkylguanine which causes G:C→A:T transition mutations. Repair by the O^6-alkylguanine-DNA alkyltransferase occurs much less efficiently in the brain, i.e. the target tissue, than in the liver and other extraneural tissue [5]. This was thought to be the mechanism of preferential induction of nervous system tumors but the CNS of mice and gerbils showed a similar repair deficiency although ENU and related agents do not have a significant neuro-oncogenic potential in these species [5]. So far, no transformation-associated gene has been identified to play a role in the evolution of ENU-induced CNS tumors. In particular, *ras* or *p53* mutations were not found in these neoplasms. In contrast, the genetic basis of ENU-induced schwannomas has been elucidated. These tumors invariably contain a T→A transversion in the transmembrane domain of the *neu* proto-oncogene [6].

3. Viral oncogenes

In an effort to identify the specific molecular changes responsible for induction of gliomas, we have established a grafting technology to directly introduce activated (viral) oncogenes into neuroectodermal precursor populations in vivo. Single cell suspensions prepared from fetal rat brains were infected with replication-defective retroviral vectors encoding oncogenes and injected into the caudoputamen of adult rats. Given the frequent finding of activated, overexpressed or deregulated tyrosine kinase oncogenes, we reasoned that it might be interesting to study the consequence of widespread activation of oncogenes belonging to the 'intimate' *src* family. These oncogenes share various structural and functional characteristics, and many of them are highly expressed in the developing nervous system and in some human and experimental neuroectodermal tumors. This family of oncogenes is still growing, and now the following members are known: c-*src*, c-*fyn*, c-*yes*, and c-*yrk*. To deregulate the activity of these tyrosine kinase proteins we exploited a viral oncogene, the middle T antigen of polyoma virus (mT). Although mT lacks an enzymatic activity, it is capable of forming a protein complex with several members of the *src* family and of upregulating their tyrosine kinase activity.

The middle T antigen of polyoma virus lacks an enzymatic activity but is capable of forming a protein complex with several members of the *src* family and of upregulating their tyrosine kinase activity. In rats carrying transplants expressing

the polyoma middle T antigen we observed endothelial hemangiomas in the graft which in 70% of the recipient animals led to fatal cerebral hemorrhage within 13–50 days after transplantation [7]. Rats that did not die from ruptured hemangiomas developed anaplastic gliomas after several months. It appears that the rapid induction of hemangiomas is induced by middle T alone, whereas delayed glioma induction requires additional genetic alterations.

Since polyoma mT activates *src*, we introduced into fetal brain cells the v-*src* oncogene of the Rous sarcoma virus. Expression of the v-*src* gene caused astrocytic and mesenchymal tumors with a 70% incidence after latency periods of 2–6 months, but no endothelial lesions. It was found by in situ hybridization that these oncogenes are expressed in cells belonging to all types represented in the graft, indicating that cell-type specific transformation is due to differential susceptibility of the respective target cell to the oncogenes, rather than selective integration or expression of the retroviral construct [7].

While retroviral vectors containing the *ras* oncogene produced a low incidence of anaplastic gliomas, v-*myc* alone was unable to induce malignant transformation. However, constructs containing *ras* plus *myc* proved very effective, causing the induction of multiple, highly anaplastic tumors within a few weeks after transplantation [8]. In this model system, we could also demonstrate the co-operation of *myc* and a chemical carcinogen (ENU) in the induction of CNS neoplasms [9]. Tumors induced by SV40 large T antigen showed a high degree of similarity with the human medulloblastoma, a malignant embryonal tumor of childhood. SV40-like sequences have indeed been observed in pediatric brain tumors but so far this has not led to the identification of the responsible viral pathogen. The highly efficient gene transfer by retroviral vectors into fetal brain transplants provides a challenging experimental strategy to study differentiation and oncogenesis in the CNS and may also become useful in assessing the organ-specific transforming activity of specific *p53* mutations observed in spontaneous and inherited human neuroectodermal neoplasms.

4. Epidemiology of human brain tumors

In Western Europe and North America, there are about 5–9 new cases of primary CNS tumors per 100 000 inhabitants each year. Approximately half of these are of glial origin. In multi-racial countries, whites are more frequently affected than persons of African or Asian descent. Males are more frequently affected than females (M/F ratio, 1.4:1) with the exception of the usually benign meningiomas.

Analytical epidemiological studies have revealed an increased risk of brain tumor development in association with certain occupations, e.g. in farmers, dentists, fire fighters, metal workers and in the rubber industry but attempts to identify a specific exposure or causative environmental agent have been unsuccessful [10]. The somewhat increased incidence of CNS neoplasms in anatomists and embalmers pointed to a possible role of formaldehyde but in an industrial setting exposure to this weak carcinogen is not associated with an increased risk [11]. Similarly, multicentre studies have not substantiated the hypothesis that occupational exposure to vinyl chloride and electromagnetic fields carries an enhanced risk of brain tumor development [12]. However, therapeutic irradiation of the brain, particularly in children, can lead to the development of malignant gliomas or primitive neuroectodermal tumors after an interval of usually 6–9 years [13].

5. Molecular genetics of human brain tumors

Some genotoxic carcinogens cause typical or even specific mutations in transformation-associated genes and thereby allow conclusions regarding the causative agent operative in the initiation of the respective neoplasms. We have, therefore, analyzed *p53* mutations as a possible potential source of information on the etiology of human CNS neoplasms.

5.1. Glioma progression

Astrocytic brain tumors (with the exception of the pilocytic astrocytoma) have an intrinsic tendency to progress towards a more malignant

phenotype and to ultimately acquire the histopathological and clinical characteristics of a glioblastoma. There is increasing evidence that astrocytoma progression reflects the sequential accumulation of genetic alterations. The identification of these events and the assessment of their roles in malignant transformation are essential for our understanding of evolution of astrocytomas and are prerequisite for therapeutic approaches at the molecular level.

Diffusely infiltrating low-grade astrocytomas (WHO Grade II) have an intrinsic tendency to progress to anaplastic astrocytoma (WHO Grade III) and glioblastoma (WHO Grade IV). This change is due to the sequential acquisition of genetic alterations, several of which have recently been identified. In low-grade astrocytomas, *p53* mutations with or without loss of heterozygosity on chromosome 17p are the principle detectable change. Anaplastic astrocytomas contain *p53* mutations at an overall incidence of 34% and, in addition, loss of heterozygosity on chromosome 19q and frequent homozygous deletion of the p16 tumor suppressor (MTS-1) gene. The most malignant astrocytic neoplasm, the glioblastoma, further shows loss of chromosome 10 and amplification of the epidermal growth factor receptor (EGF-R) gene at overall incidences of 66 and 34%, respectively [14,15].

5.2. Type and distribution of p53 mutations in astrocytic brain tumors

In sporadic astrocytic brain tumors, *p53* mutations are mainly located in the highly conserved region of the gene, with clusters at codons 175, 248 and 273 [14,16]. These codons are among the 6 hot spots found in a variety of human tumors [17–19]. Among *p53* mutations identified in astrocytic brain tumors, G:C→A:T transitions are most frequent and they are predominantly located at CpG sites (Table 1). These patterns of *p53* mutations are similar to those in colon cancer, sarcomas and lymphomas but different from those in non-small-cell lung cancer (SCLC) and liver cancer which are considered to be associated with tobacco-related carcinogens and aflatoxin B1, respectively [14,15,18]. The transition mutations at CpG sites can best be explained as being due to the deamination of 5-methylcytosine residues and considered to be endogenous, i.e., not caused by genotoxic environmental carcinogens. In summary, no specific mutations or mutational hot spots, which could be suggestive of environmental carcinogens operative in the etiology, were found in human brain tumors.

5.3. Brain tumors associated with p53 germline mutations

Until now, 91 families with germline *p53* mutations have been reported, with a total of 475 tumors in affected family members [unpublished]. Breast cancer developed most frequently, followed by sarcomas, brain tumors, and neoplasms of the gastro-intestinal tract. Brain tumors occurred in 39 out of 91 pedigrees (43%). Of the 57 brain tumors recorded, 25 (45%) were not specified histologically. Of the remaining, 22

Table 1
Frequency of G:C→A:T transition mutations in the *p53* gene in human brain tumors

	Mutations at G:C (%)			G:C→A:T transition at CpG sites (%)
	→A:T	→T:A	→C:G	
Brain	73	5	3	52
Colon	79	0	3	67
Sarcoma	66	7	7	53
Lymphoma	57	4	4	47
Germline mutations	64	13	2	49
Lung:non-SCLC	20	57	13	10
Liver	16	74	5	0

Adapted from Ohgaki et al. [15] and Hollstein et al. [18].

(73%) were of astrocytic origin. This, together with the observation of frequent *p53* mutations in sporadic astrocytomas [14], strongly supports the view that loss of *p53* function carries a risk of malignant transformation for astrocytes more than any other cell type of the human nervous system.

Among *p53* germline mutations in 91 families, point mutations causing amino acid substitutions were most frequent (81%). Among these, G:C→A:T transition mutations prevailed and 77% of them were located at CpG sites (Table 1). Thus, the type of *p53* germline mutations was similar to that found in sporadic astrocytic brain tumors, suggesting that these germline *p53* mutations may also have evolved endogenously rather than as a consequence of interaction with environmental carcinogens.

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The use of transgenic mice for studying mutagenicity induced by 1,3-butadiene

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Abstract

The availability of in vivo transgenic mouse mutagenesis assays provides the opportunity to study the induction of mutations that reflects the integration of pharmacokinetics, biotransformation, DNA repair responses, and tissue susceptibilities. Transgenic mice containing in vivo marker genes for mutagenicity within shuttle vectors also permit the determination of the resulting mutational spectra in tissues of mice following exposure to mutagens and carcinogens. Exposure of B6C3F$_1$ *lacI* transgenic mice to the rodent carcinogen 1,3-butadiene by inhalation results in an increase in the overall *lacI* mutant frequency in bone marrow with an increased frequency of point mutations at A:T base pairs. These studies demonstrate the utilization of transgenic mouse models for establishing linkages between exposure to carcinogens, internal tissue dose, and in vivo mutational events.

Keywords: *lacI*; 1,3-Butadiene; Transgenes; Mutation spectrum

1. Introduction

There is a need to improve the process of carcinogen identification and to further our understanding of human risk from exposure to carcinogens. Transgenic models represent new experimental tools to address these needs. Transgenic animals are constructed using molecular genetics techniques to produce animals that have functional genes added to the genome, contain marker genes to assess in vivo effects, or have alterations of specific genes believed to be involved in disease processes. Transgenic model systems should improve detection of mutagens and potential carcinogens, aid in developing predictive systems for carcinogenic activity that are knowledge based, and reduce animal usage [1]. In addition, transgenic model systems will provide improved biomedical tools for toxicological assessments, chemically induced mechanisms of disease, and exposure-dose extrapolations [2].

Numerous transgenic animal models have been developed for studying cancer processes [3]. At present, many of these animals are used for studies of carcinogenesis, since they permit the assessment of gene mutations induced in vivo, contain activated oncogenes, or contain inactivated tumor suppressor genes that are believed to be involved in tumor development. General conclusions from these animal models include the focal nature of the observed tumors and their variable latency periods, a cooperation/interaction between transgenes in tumor development, a

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variable and often strong strain influence on transgene-induced neoplastic responses, and a differential response to carcinogen exposure. Ultimately, transgenic animals may aid in identifying critical genetic alterations required for specific tumor formation and how chemical carcinogens can act to modulate these steps. As advances are made in the use and characterization of transgenic models, the future challenge is to appropriately use the information in improving estimations of human health risks. Significant progress in assessing in vivo mutations following carcinogen exposure has been made with transgenic mouse models. This review demonstrates the utility of one such in vivo mutagenesis model for assessing carcinogen exposure-dose-response linkages.

2. Transgenic mice for studies of in vivo mutagenesis

Transgenic mouse models containing a λ phage shuttle vector with bacterial genes (e.g., *lacZ* or *lacI*) inserted as mutational targets integrated into the mouse genome are being used as in vivo gene mutation assays [4,5]. These in vivo transgenic mouse mutagenicity assays provide unique opportunities for studying the induction of tissue-specific mutations that reflect the integration of pharmacokinetics, biotransformation, DNA repair, and tissue susceptibility. Such endogenous processes, likely to be modulators of in vivo mutagenicity, are not readily reproduced using in vitro cell culture mutagenicity assays. In addition, the relationship between chemically induced in vivo cytotoxicity, cell proliferation and mutagenicity can also be examined in transgenic mice [6]. The mutated *lac* transgenes can be sequenced to assess the types and specificity of mutations and the role of clonal expansion in the induction of in vivo mutagenicity [7].

Our laboratory is utilizing transgenic mice to establish linkages between exposure to the rodent carcinogen 1,3-butadiene (BD), internal target tissue dose, and in vivo mutation [7,8]. BD is carcinogenic and genotoxic in $B6C3F_1$ mice following inhalation exposure [9,10]. We have exposed $B6C3F_1$ *lacI* transgenic mice to BD by inhalation and have determined the in vivo mutagenicity and the mutational spectra at the *lacI* transgene recovered from the bone marrow.

3. In vivo *lacI* mutant frequency in BD-exposed mice

Male $B6C3F_1$ *lacI* transgenic mice were exposed to 62.5, 625, and 1250 ppm BD for 4 weeks (5 days/week and 6 h/day), and animals were euthanized 2 weeks after the final exposure. DNA was extracted from the bone marrow, and mutagenicity at the *lacI* transgene was determined [5]. The resulting *lacI* mutants were subjected to DNA sequence analysis to determine the BD-in vivo mutational spectrum and to assess the contribution of in vivo clonal expansion to the *lacI* mutant frequency [7,8].

BD was mutagenic in the bone marrow following inhalation exposure [8]. A concentration-dependent increase in the *lacI* mutant frequency occurred in the bone marrow, with up to a 4-fold increase above that for air-control mice at 625 ppm and 1250 ppm BD (Table 1). The *lacI* mutants counted from each animal were collected. Following DNA sequence analysis, the type of mutational event and its position within the *lacI* gene was recorded to determine the mutational spectra.

4. In vivo *lacI* mutational spectrum in air-control and BD-exposed $B6C3F_1$ *lacI* transgenic mice

4.1. Background mutational spectra in bone marrow

Of the 56 *lacI* mutants isolated from air-control animals, 45 (80%) were considered to be independent mutational events by DNA sequence analysis of the *lacI* transgene. *lacI* mutants with identical mutations isolated from the same animal were assumed to be mutant siblings and were considered to be one in vivo mutational event (Table 2). Single base substitutions accounted for 41/45 (91%) of the background mutations, with base substitution at G:C base pairs compromising 39/41 (95%). Of 28 G:C → A:T transitions, 23 (82%) occurred at CpG

Table 1
lacI$^-$ mutant frequency in bone marrow of B6C3F$_1$ transgenic mice following inhalation exposure to BD

Animal no.	BD exposure (ppm)	*lacI*$^-$ plaques/total plaques	*lacI*$^-$ mutant frequency ($\times 10^{-5}$)
2	0	33/887 005	3.7
3	0	30/754 006	4.0
4	0	33/854 421	3.9
		mean ± S.D.	3.8 ± 0.1[a]
7	62.5	26/526 463	4.9
8	62.5	37/503 254	7.4
9	62.5	49/522 958	9.4
		mean ± S.D.	7.2 ± 2.2[a]
11	625	146/658 599	22.2
12	625	80/773 213	10.3
13	625	100/740 697	13.5
		mean ± S.D.	15.3 ± 6.1[a]
16	1250	90/549 709	16.4
18	1250	59/544 276	10.8
20	1250	65/496 665	13.1
			13.4 ± 2.8[a]

[a]Mean ± S.D.

Table 2
lacI mutations recovered from the bone marrow of B6C3F$_1$ *lacI* transgenic mice: air controls and groups exposed to 625 and 1250 ppm BD

Point mutations	Air-control mutations (%)	Mutations at 625 ppm (%)	Mutations at 1250 ppm (%)
At G:C base pairs			
GC→AT	28 (62)	11 (42)	19 (39)
GC→TA	9 (20)	3 (12)	8 (16)
GC→CG	2 (4)	4 (15)	3 (6)
At A:T base pairs			
AT→GC	1 (2)	4 (15)	5 (10)
AT→CG	1 (2)	0	1 (2)
AT→TA	0	2 (8)	4 (8)
Multibase alterations			
Tandem change	0	0	1 (2)
Insertions	1 (2)	0	4 (8)
Deletions	3 (7)	2 (8)	4 (8)
Total	45	26	49

The in vivo mutant frequency determined for the data are reported in Sisk et al. [8].

dinucleotides. G:C → A:T transitions at CpG dinucleotides are a common mutation involved in the in vivo background *lacI* mutational spectrum, and likely result from the deamination of methylated cytosines at 5′-CpG-3′. Only 2/45 (4%) of the mutations recovered in the air-control animals were base substitution mutations at A:T base pairs.

4.2. Mutational spectra in BD-exposed mice

A total of 54 *lacI* mutants isolated from bone marrow of animals exposed to 625 ppm BD were analyzed by DNA sequencing (Table 2). Of 54 mutants analyzed, 26 (48%) were independent mutational events. In 1 animal from the BD-exposed group (animal no. 11), mutant siblings accounted for 80% of all the mutants analyzed,

i.e., only 20% of the mutants analyzed from animal 11 were independent. As observed in air-control animals, 24/26 (92%) of the mutants analyzed contained single base substitution mutations. In contrast to the spectrum of point mutations observed in air controls, 6/26 (23%) of the point mutations determined in BD-exposed mice occurred at A:T base pairs.

A total of 74 *lacI* mutants isolated from the bone marrow of *lacI* transgenic mice exposed to 1250 ppm were sequenced and analyzed for the presence of mutations. Of the 74 mutants analyzed, 49 (66%) were considered to be independent mutational events (Table 2). Of the *lacI* mutations recovered from the bone marrow, 40/49 (82%) were point mutations; 30/49 (61%) were point mutations at G:C base pairs, while 10/49 (20%) were point mutations at A:T base pairs. The increases observed for point mutations at A:T base pairs in the present work are consistent with those previously observed in *lacI* mice exposed to 625 ppm BD [8]. Examination of the DNA sequence context of the A:T site mutations demonstrated the presence of the dinucleotide 'AC' in 10/15 (67%) of the sites of A:T base pair mutations in BD-exposed animals.

There was no significant difference in the distribution of mutational types between the 625 ppm and 1250 ppm BD-exposed group (ANOVA $P < 0.05$). There were no apparent differences in the frequency of complex mutations (deletions, insertions, and tandem changes) between air-control and BD-exposed *lacI* mice. There was an increase in point mutations occurring at G:C and A:T base pairs with exposure of BD (Fig 1). However, only the frequency of point mutations at A:T base pairs for 625 ppm and 1250 ppm BD was shown to be significantly greater than the air controls ($P < 0.01$). The frequency of mutations at A:T base pairs in BD-exposed *lacI* mice was more than 10-fold over the air controls. The associated P value for an increased frequency of mutations at G:C base pairs relative to air controls was $P = 0.53$ at the 1250-ppm exposure group.

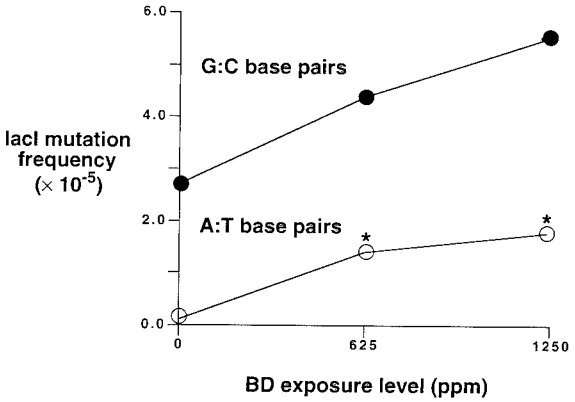


Fig. 1. Distribution of the *lacI* mutation frequency among the total frequency of mutations occurring at G:C base pairs (●) and A:T base pairs (○). The data for 625 ppm BD are from Sisk et al. [8]. The contribution of each mutational type to the *lacI* mutation frequency was calculated from the mutation frequency and the percentage of point mutations occurring at either G:C or A:T base pairs. The points plotted are the mean values for these mutational types for each exposure group. *Significantly greater than air controls ($P < 0.05$) by ANOVA. At 1250 ppm for G:C base pairs, $P = 0.053$.

5. Discussion

Transgenic mice containing shuttle vectors provide a unique opportunity for assessing the in vivo mutant frequency and the resulting mutational spectra in tissues of mice exposed to carcinogens. We used exposure levels known to induce tumors and the same strain of mouse ($B6C3F_1$) utilized in the carcinogenicity bioassay of BD to assess the in vivo mutagenicity of BD. Studies on the in vivo mutational spectra of BD can contribute to the understanding of mechanisms of BD-induced mutations and the role of mutagenicity in the carcinogenicity of BD. Specific point mutations in the K-*ras* oncogene [11] and the *p53* tumor suppressor gene [12] occur in BD-induced tumors in $B6C3F_1$ mice, indicating that specific gene mutation could be a likely step in the induction of tumors in mice exposed to BD. Although these studies primarily focus on gene mutation events, BD clearly can also induce a variety of chromosomal alterations in mice and this can influence the formation of tumors [10].

This work has demonstrated an increased frequency of point mutations at A:T base pairs at

the *lacI* transgene in B6C3F$_1$ mice exposed to BD. The origin of the increased frequency of mutations at A:T base pairs in BD-exposed *lacI* mice is at present uncertain [7,8]. An increased frequency of mutation at A:T base pairs also occurs in *hprt* mutant T lymphocytes isolated from B6C3F$_1$ mice exposed to BD [13], and mutations at A:T base pairs in the tumor suppressor gene *p53* occur in BD-induced lung tumors [12].

There was a slight increase in the frequency of point mutations at G:C base pairs at 1250 ppm BD. Therefore, it is apparent that BD induces point mutations at both G:C and A:T base pairs. Although guanosine and adenosine adducts have been reported following in vitro reactions of BD metabolites with DNA [14,15], a comprehensive evaluation of BD-derived DNA adducts either in vitro or in vivo is not available. Because BD bioactivation in mice produces 2 genotoxic metabolites with different potencies, it is necessary to evaluate the mutagenicity and mutational spectrum of each metabolite independently to assess its role in the in vivo mutagenicity and mutational spectrum of the parent compound BD.

Determination of the in vivo *lacI* mutant frequency in the tissues of transgenic mice is an approach to assessing tissue-specific mutagenicity and mutational spectra following carcinogenic exposures. The *lacI* mutant frequency obtained is likely to be modulated by a number of factors, including tissue-specific concentrations of mutagenic metabolites, the quantities and sites of different adducts, tissue-specific DNA repair pathways, and cellular proliferation rates in different tissues. The in vivo proliferation of a mutated cell will give rise to mutant siblings and a clonal expansion of the initial mutational event. Mutant siblings and their clonal expansion can also affect the in vivo *lacI* mutant frequency. However, DNA sequence analysis can be used to assess the contribution of mutant siblings to the *lacI* mutant frequency [7,8].

These studies demonstrate an increased frequency of in vivo point mutations occurring at A:T base pairs following BD inhalation exposures in B6C3F$_1$ *lacI* transgenic mice. Since BD also induces a variety of in vivo chromosomal alterations in mice, these data indicate that BD can induce in vivo genotoxicity by several mechanisms. How these differing pathways of genotoxicity and their induction by specific BD metabolites contribute to the induction of tumors in BD-exposed mice is uncertain.

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Genetics of liver tumor susceptibility in mice

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Abstract

A good experimental model of genetic predisposition to hepatocellular tumors is the murine strain C3H. These tumors share morphologic similarities with human hepatocellular tumors. After a treatment with a single small dose of chemical carcinogen, the C3H mice show a high susceptibility to the growth of hepatocellular neoplastic lesions, that reach a volume >100-fold as compared to the corresponding lesions of genetically resistant strains. Genetic linkage analysis experiments were conducted in 2 different crosses, with the C3H as one of the parental strains, and the other parental strains being represented by mice genetically resistant to hepatocarcinogenesis (A/J, *M. spretus*). Six different regions, on chromosomes 2, 5, 7, 8, 12, and 19 showed a significant linkage with hepatocellular tumor development. These results provide the genetic basis for the strain variations seen in susceptibility to hepatocarcinogenesis, indicating polygenic inheritance of this trait.

Keywords: Liver carcinogenesis; Mice; Susceptibility; Resistance

1. Introduction

The C3H and CBA murine inbred strains present a high spontaneous incidence of hepatocellular tumors, whereas other strains (A, BALB/c, C57BL/6, SWR) develop spontaneous liver tumors at a very low rate (Table 1). Both CBA and C3H strains arose in 1920 from a single cross of the Bagg and DBA lines [1,2]. This common origin, along with the subsequent phylogenetic analysis, confirms the close relationship between CBA and C3H mice, [3], and suggests that the same genetic alteration(s) are responsible for the high susceptibility to hepatocarcinogenesis (HCC) evident in both strains. Although the C3H strain exhibits a high incidence of spontaneous HCC, it does not show any sign of liver cirrhosis or liver dysfunction that could be associated with its genetic susceptibility.

Susceptibility to liver carcinogenesis is a dominant or a semi-dominant genetic trait. In fact, F1 mice derived from parents whom are both susceptible to HCC (CBA × C3H) retain the parental susceptibility. F1 hybrids between susceptible and resistant mice are susceptible. F1 progeny from mice resistant to HCC are resistant, except for a single case where the 129 × DBA/2 mice had an increased incidence of liver tumor with respect to both parents (Table 1).

One outbred strain, the CF-1, is also characterized by a high susceptibility to spontaneous and chemically induced HCC [4]. However, outbred

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Table 1
Murine inbred strains with different susceptibility to the development of spontaneous hepatocellular tumors

Strain/hybrid[a]	% Liver tumor incidence (range)	Duration of the experiment (range)[b]	Reference
129/J	0	LS	[30]
(129 × DBA/2)F1	19	LS	[30]
A/He	0	12 months	[31]
(A/J × C57BL/10)F1	2	LS	[30]
BALB/c	0–9	1 year-140 weeks	[5,32]
C3H	14–67	1 year–LS	[5,6,30,33,34]
(C3HeB/FeJ × A/J)F1	8	142 weeks	[7]
C57BL	0–3	1 year–LS	[5,6,30]
(C57BL/6J × BALB/c)F1	0–4	80–110 weeks	[8,35]
(C57BL/6J × C3H)F1	7–44	90–142 weeks	[6–8,36]
CBA	15–23	1 year–LS	[5,30]
(CBA × C3H)F1	24	1 year	[5]
(CBA × C57BL/10)F1	7	LS	[30]
DBA/2J	1	LS	[30]
IF	0	1 year	[5]
LP/J	6	LS	[30]
(LP × 129)F1	7	LS	[30]
(LP × C57BL/10)F1	7	LS	[30]
(LP × DBA/2)F1	9	LS	[30]
SWR	0	100 weeks	[6]

[a] Male mice only.
[b] LS, lifespan.

strains in general are much less suitable for genetic studies than inbred strains.

Strains that are genetically susceptible to spontaneous hepatocellular tumor development are also particularly susceptible to chemical carcinogen-induced HCC. Indeed, they develop earlier and more liver tumors than genetically resistant ones [5–10].

2. Phenotype analysis

In recent years, identification of the chromosomal localization of genes responsible for important human inherited diseases was the first step toward positional cloning and characterization of these genes.

In the case of liver carcinogenesis, susceptibility is not a discrete trait but rather refers to quantitative values. For this reason, mapping the gene(s) responsible for high susceptibility to HCC requires the definition of quantitative indexes of tumor susceptibility. Quantitative estimates of liver neoplastic lesions are performed by stereological methods that allow mathematical extrapolation to 3 dimensions from 2-dimensional data. These methods allow estimation of tumor frequency and size. This is important because the susceptible phenotype may result from a genetic effect on either initiation (high tumor number) or on progression (high tumor size) steps.

Quantitative indexes of susceptibility that we are currently using include the number of nodules/cm^3 liver (N/cm^3), the mean volume of nodules, and the percentage of liver volume occupied by nodules (V%). N/cm^3 represents an estimate of tumor frequency, the mean volume of nodules represents an estimate of tumor size, and V% represents an estimate of both tumor frequency and size, (i.e., it represents the volume fraction of liver occupied by tumors). Susceptibility to HCC among strains showed wide variations, depending on the parameters examined, e.g., tumor incidence, tumor multiplicity, and tumor size [6,9,11–13].

Indeed, we and other groups have previously reported that genes affecting susceptibility to murine HCC control the progression (tumor

size) but not the frequency (tumor number) of carcinogen-induced liver tumors [9,11,14]. Accordingly, microscopic liver foci induced by the carcinogen ethylnitrosourea (ENU) had about a 3-fold higher labelling index in C3H/HeJ than in C57BL/6J mice [11]. However, another report showed no correlation between labelling index and susceptibility [15].

3. Genetic analysis

Drinkwater and Ginsler [10] have proposed a 2-loci (named *hepatocarcinogen sensitivity*, *Hcs*) model to explain the difference in susceptibility to *N*-nitrosodiethylamine (NDEA) and ENU-induced liver carcinogenesis between the C3H/He and C57BL/6J strains. One locus accounted for about 80% of the greater sensitivity of C3H/He mice, suggesting that the other locus plays a minor role.

In order to identify the loci involved in inherited predisposition to murine HCC, we performed a liver carcinogenesis experiment in an F2 cross between the resistant A/J and the susceptible C3H/He strains. The resulting AC3F2 male mice were treated with a single dose of urethane and then kept under observation until 40 weeks of age.

The percentage of liver volume occupied by nodules (V%) was used as a quantitative index of susceptibility to liver carcinogenesis. This index differs from one parental strain to the other by a factor >100 [14], making the elucidation of genes affecting the susceptibility trait possible. We investigated the segregation of liver tumor susceptibility with genetic markers dispersed over the entire array of autosomes.

Three genomic regions important in determining quantitative variation in liver tumor susceptibility were identified on chromosomes 7, 8, and 12. We named these loci *Hcs*1, *Hcs*2, and *Hcs*3, respectively [16] (Table 2). *Hcs*1 (chromosome 7) and *Hcs*2 (chromosome 8) derived from the C3H/He strain, and *Hcs*3 was unexpectedly associated with the A/J allele.

In this genetic linkage study, the low genetic divergence and the consequent low degree of polymorphism between the parental strains made it difficult to identify genetic markers in any of the chromosomal regions. To improve the possibility of finding polymorphic markers, we repeated the experiment in an interspecific cross that included the *M. spretus* mice, a strain evolutionarily distant from laboratory mice, which offers a great level of allelic polymorphism. Thus, we crossed the liver tumor susceptible C3H/He strain with the *M. spretus* strain. Since we had no information on the susceptibility

Table 2
Murine loci containing putative hepatocellular tumor susceptibility/resistance genes, as detected by genetic linkage studies

Locus name	Chromosome (cM, range)	Human homologous region(s)	Genetic cross(es)	Strain derivation of the susceptibility allele	Reference
*Hcs*1	7 (20–40)	multiple	(A/J × C3H/He)F2	C3H/He	[16]
*Hcs*2	8 (45–56)	16q	(A/J × C3H/He)F2	C3H/He	[16]
*Hcs*3	12 (70–73)	14q	(A/J × C3H/He)F2	A/J	[16]
*Hcs*4	2 (76–94)	20q	(C3H/He × *M. spretus*) × C57BL/6J	C3H/He	[18]
*Hcs*5	5 (38–45)	4q	(C3H/He × *M. spretus*) × C57BL/6J	C3H/He	[18]
*Hcs*6	19 (50–75)	10q	(C3H/He × *M. spretus*) × C57BL/6J	*M. spretus*	[18]
*Hcr*1	4 (50–75)	1p	(DBA/2J × C57BL/6J)F2	C57BL/6J	[19]
*Hcr*2	10 (31–51)	multiple	(DBA/2J × C57BL/6J) × C57BL/6J (DBA/2J × C57BL/6J)F2 (DBA/2J × C57BL/6J) × C57BL/6J	C57BL/6J	[19]

of the *M. spretus* strain to HCC, we made the cross between the (C3H/He × *M. spretus*)F1 mice and the C57BL/6J strain, which is resistant to HCC, but whose F1 hybrids with the C3H/He strain are genetically susceptible [6,17]. The resulting testcross individuals (named HSB mice) were analysed to detect the association between susceptibility to HCC and a set of molecular markers.

The genetic linkage analysis indicated that 3 *Hcs* loci were significantly associated with the liver tumor susceptibility (Table 2). *Hcs*4 was located on chromosome 2; a central region of chromosome 5 contained the *Hcs*5 locus, and a distal region of chromosome 19 contained the locus *Hcs*6 [18].

We found that a portion of the tumor susceptibility trait was associated with inheritance of the C3H/He allele at *Hcs*4 and *Hcs*5 loci, and from inheritance of the *M. spretus* allele at the *Hcs*6 locus. However, this kind of analysis cannot determine whether the strain whose genomic region is associated with susceptibility carries a susceptibility gene or whether the effect is due to the presence of a resistance gene contributed by the other strain. The tumor frequency (N/cm^3) showed no significant linkage with any of the 232 genetic markers, indicating that it is not genetically determined, as previously suggested [9,11,14].

Interestingly, the loci controlling liver tumor development that we previously mapped in the AC3F2 cross, i.e., the loci on chromosomes 7, 8 and 12, were not detected in the present cross. Since the locus on chromosome 12, derived from the A/J strain that was absent in the present cross, we did not expect to confirm this locus in the HSB mice. However, the loci on chromosomes 7 and 8 previously found in the AC3F2 mice and derived from the C3H/He parent were not identified in the present analysis. Moreover, we tested back on AC3F2 mice the genetic markers of the chromosomal regions containing *Hcs* loci identified in the HSB cross. The corresponding regions in the AC3F2 sample did not contain *Hcs* loci [18].

A possible explanation for the discrepancies in the positioning of *Hcs* loci in the 2 crosses may be due to the different parental strains used. Indeed, if both parental alleles contain the same *Hcs* locus, no differences can be detected in the phenotype of the test sample, since linkage analysis can only detect *Hcs* loci when they are retained in just 1 of the 2 segregating alleles. Therefore, using *M. spretus* instead of the A/J as one of the parents, we have identified new regions involved in the determination of susceptibility to HCC. More recently, 2 HCC resistance loci, *Hcr*1 and *Hcr*2, carried by the DBA/2J mice, have been mapped on chromosomes 4 and 10 [19].

The present findings indicate that multiple unlinked genetic loci control liver tumor development in mice. Thus, inherited susceptibility to liver tumor development would represent a new genetic model for tumor predisposition, characterized by the interaction of multiple loci, that, taken alone, provide a relatively low contribution that is insufficient to reveal the tumor susceptible phenotype (phenotypic variance ranging between 10 and 15%).

4. Possible mechanisms of genetic predisposition

4.1. Loss of heterozygosity (LOH)

Tumors from subjects with inherited familial predisposition to different types of cancer, including retinoblastoma, breast cancer, and familial adenomatosis polyposis (FAP), are characterized by frequent LOHs at the chromosomal regions where the cancer predisposition loci have been mapped. Because in these subjects one allele of the cancer predisposition gene carries a germ-line mutation that leads to a loss of gene function, LOH represents a mechanism of inactivation of the remaining functional allele.

Several studies have reported a high frequency of loss of LOH in human HCCs at specific chromosomal regions. The chromosomes most frequently involved are 4p15-q21, 8p22, 11p15-p13, 13q12-q32, 16q22-q24, and 17p13. These findings indicate that putative oncosuppressor genes play an important role in human HCCs. Two of the human chromosomal regions frequently affected by LOH (4p, 16q) have their homologous counterparts on mouse chromosomes 5 and 8, where *Hcs*5 and *Hcs*2, respective-

ly, have been mapped [16,18]. However, a genome-wide search for LOHs in mouse primary HCCs showed that LOHs are very rare in any chromosomal region, including those where *Hcs* loci have been positioned [20,21].

These results indicate that the loss of the normal function does not presumably represent a mechanism of generation of *Hcs* alleles. However, cell cultures of mouse HCCs showed instead a frequent occurrence of LOHs on chromosome 4 [21], in a region where a hepatocellular tumor resistance locus (*Hcr*1) has been mapped [19].

4.2. Genetic instability

The molecular basis for predisposition to hereditary non-polyposis colon cancer (HNPCC) has been understood through the observation of its mutator phenotype. Widespread deletions or insertions at mono-, di-, and trinucleotide repeat sequences were found in a majority of colon tumors from individuals with HNPCC [22]. This phenotype has also been detected in other types of human tumors and in mouse and rat colon tumors [23–25]. However, our recent observations revealed that in murine hepatocellular carcinomas short repeated sequences are genetically stable and do not show any increase or decrease in the number of repeat units. Moreover, as shown in Fig. 1, a highly unstable minisatellite showed no size alterations in tumors compared with normal tissue [20].

4.3. Ha-ras gene mutations

The frequency of Ha-*ras* mutations is about 10-fold higher in liver tumors developed in genetically susceptible strains than in the corresponding tumors of the resistant mice [26,27]. Ha-*ras* mutations appear as an early event in the pathogenesis of mouse liver tumors, because they are observed in microscopic preneoplastic lesions [28]. Therefore, Ha-*ras* mutations may represent a molecular alteration that could lead to the higher growth rate of liver tumors in the genetic susceptible than in resistant strains. Obviously, the Ha-*ras* gene does not represent one of the *Hcs* genes, because mutations are somatically acquired in liver tumors and are not present in the germ-line of the C3H mice.

To identify the genetic determinants of Ha-*ras* mutations in liver tumors, we assessed the presence of Ha-*ras* mutations at codon 61 in liver tumors induced by urethane in AC3F2 and HSB mice, 2 genetic crosses that we have used to map *Hcs* loci. In the same crosses we have investigated the possible linkage of Ha-*ras* mutations with genetic markers. However, in both crosses we have not found any significant linkage between the inheritance of any genetic locus and the presence of Ha-*ras* mutations in tumors (data not shown). The fact that the linkage failed to reveal an association between Ha-*ras* mutations (a discrete, not quantitative, trait) and genetic markers may indicate the possible polygenic control of Ha-*ras* mutations. Therefore, these results do not rule out the genetic determination of Ha-*ras* mutations.

5. Conclusions

Genetic susceptibility in the murine model of inherited predisposition to HCC is characterized by a rapid growth of neoplastic nodules and by the consequent high risk of HCC, whereas liver

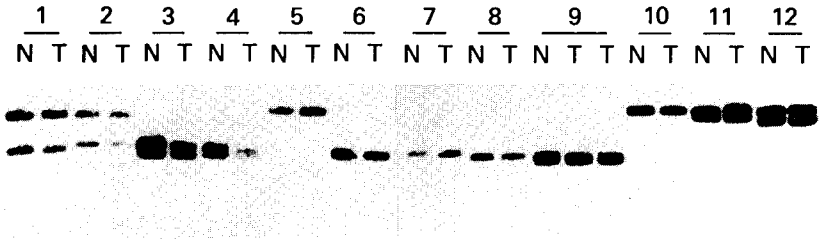


Fig. 1. Highly unstable minisatellite showing no size alterations in the murine hepatocellular tumors compared with the normal tissue. Inter-individual instability is apparent. Southern bolt hybridization of $(GGGCA)_3$ oligonucleotide to DNAs from control tissue and urethane-induced liver tumors of male HSB mice. (Reprinted by permission of John Wiley & Sons, Inc.).

tumor frequency is not genetically determined. Multiple unlinked genetic loci control liver tumor development, indicating that inherited predisposition to HCC constitutes a polygenic model of cancer susceptibility. The distinct genetic elements, taken alone, provide a low contribution to the trait, but their epistatic or pleiotropic effects produce the susceptible phenotype.

At present, the genetic and biochemical mechanisms responsible for the genetic susceptibility to HCC are not known. The frequent presence of Ha-*ras* mutations in liver tumors of mice genetically susceptible to HCC may represent one of the mechanisms by which these tumors grow faster than the corresponding tumors developed in genetically resistant strains.

The mouse liver tumor system is important for the risk assessment of potential carcinogenicity of chemicals to humans, since the genetically susceptible B6C3F1 mouse is used in the bioassay of chemicals for their carcinogenicity under the National Toxicology Program in the USA. The understanding of the mechanisms responsible for genetic susceptibilty to HCC of the C3H/He strain, and the analysis of their relevance for the pathology of human HCCs, may provide a strong scientific basis to judge the results of the carcinogenicity bioassays [29].

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Hereditary renal cell carcinoma in the Eker rat: a unique animal model for the study of cancer susceptibility

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Abstract

A class of genes, the so-called tumor suppressor genes or anti-oncogenes, was originally identified as being responsible for germ-line transmission of cancer susceptibility in humans. Tumor suppressor genes are recessive at the cellular level with respect to oncogenesis but often manifest as dominantly inherited familial cancer syndromes. This type of cancer syndrome arises in the Eker rat due to a genetic defect in the tuberous sclerosis 2 (*Tsc2*) gene. The Eker rat familial cancer syndrome serves as a unique animal model in which to study the molecular pathways of renal tubular epithelial carcinogenesis as well as a valuable tool for studies that examine how chemical carcinogens interact with cancer susceptibility genes.

Keywords: Renal carcinoma; Suppressor gene; Cancer susceptibility

1. Eker rat familial cancer syndrome

Although human hereditary cancer accounts for only a small percentage of the annual cancer incidence, it nonetheless plays a critical role in our understanding of the genetic etiology of oncogenesis. In humans, inherited predisposition to cancer occurs as a result of both recessively and dominantly inherited disorders. Recessively inherited predispositions to familial cancer, such as xeroderma pigmentosum, are generally disorders of DNA repair and the associated chromosomal breakage. These diseases predispose to mutations but do not themselves lead to identification of the gene loci in which alterations leading to cancer occur. The majority of hereditary cancer syndromes are dominantly inherited. Such predispositions have been shown to occur as a result of heterozygous defects in tumor suppressor genes. These mutations are recessive, but the probability that the remaining normal allele will become inactivated (by a variety of mechanisms) is sufficiently high that tumors develop in the majority of gene carriers. The result is a pattern of inheritance in which the susceptible phenotype appears dominant.

Eker and Mossige described a dominantly inherited cancer syndrome in rats in which bilateral multicentric renal cell carcinoma (RCC) develops at an early age [1,2]. RCC in these animals was found to arise as part of a cancer syndrome in conjunction with the development of reproductive tract leiomyomata and vascular neoplasms [3]. The occurrence of multiple

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specific tumors of diverse tissue types has been commonly associated with human familial cancer syndromes. Genetic analysis of Eker rats showed that the familial tumors were due to an alteration in a single gene, which caused heterozygote carriers of the mutation to develop RCC with complete penetrance by 1 year of age [4]. The homozygous condition resulted in embryolethality at approximately 10 days of gestation, demonstrating the importance of the gene in development.

Recently, the inherited mutation in the Eker rat was cloned using a positional cloning-candidate gene approach [5]. The cancer susceptibility gene was found to be the rat homologue of the tuberous sclerosis 2 (*Tsc2*) gene and the mutation involved a 6.3-kb insertion within an intron of the gene [6]. It has been postulated that the Eker mutation results in functional loss of *Tsc2* and aberrant transcription, leading to the loss of the *rap*1GAP-like domain, contributing to carcinogenesis. The cellular effects of the Eker mutation provide strong evidence that rat *Tsc2* functions as a tumor suppressor gene. Normal kidneys of heterozygote Eker carrier animals express both normal and abnormal *Tsc2* mRNA, but primary tumors and tumor-derived cell lines exhibit only the mutant *Tsc2* transcript. This finding supports the concept that both copies of the normal gene must be disrupted before renal tumorigenesis occurs.

2. Cellular and molecular studies of hereditary rat renal cell carcinoma

Animal models of carcinogenesis have been widely used to provide information on molecular alterations that occur in various types of cancer. These models provide a reproducible and readily available source of tumor material and allow access to tumors at all stages of development. Both normal and neoplastic cells from these animal models can often be explanted in vitro and cell lines established from these tissues, making it possible to design an approach for studying the molecular biology of these tumors that combines in vivo and in vitro techniques. The use of animal models as surrogates for the study of human neoplasms must be based on an understanding of the similarities and differences in specific tumor development between the 2 species.

Rats have provided a useful animal model for the study of human RCC due to a number of cellular, molecular, and phenotypic similarities. One important similarity between human and rat RCC is overexpression of transforming growth factor alpha (TGF-α). Normal rat proximal tubule epithelial cells do not express significant amounts of TGF-α, but this growth factor is overexpressed in both primary Eker rat RCC and tumor-derived cell lines [7]. In human RCC, overexpression of TGF-α has also been reported and acts as an autocrine growth factor for this neoplasm. Eker rat and human RCC share additional similarities. Tumors and tumor-derived cell lines do not contain alterations in the p53 tumor suppressor gene, as determined by reverse transcriptase, polymerase chain reaction and direct sequencing [8]. Activation of H-, K-, and N-*ras* oncogenes is also not observed in Eker rat RCC [9]. The absence of alterations in p53 and *ras* oncogenes is analogous to what has been reported in human RCC.

Recently we examined the sequence, expression pattern, and presence of mutations in the von Hippel-Lindau (VHL) gene in neoplasms and tumor-derived cell lines from Eker rats [10]. Unlike the situation in human RCC in which the VHL tumor suppressor gene is commonly altered, no mutations were found in the rat homologue of the gene. The genetic alteration in *Tsc2* is a critical event in the development of hereditary RCC in the rat. The importance of this particular gene in man is presently unknown, although there are reports of a low frequency of kidney tumors in tuberous sclerosis patients. Development of tubular cystic lesions is relatively common in human tuberous sclerosis patients and is a common feature of renal tubular tumors in Eker rats. Interestingly from a comparative oncology point of view, hereditary RCC in German shepherd dogs is associated with a phakomatosis similar to human tuberous sclerosis, although the genetic defect has not yet

been elucidated. Differences between rat and human RCC with respect to VHL and *Tsc2* gene alterations warrant additional investigation.

Eker rats provide a useful tool in which to dissect the molecular mechanisms underlying tubular epithelial carcinogenesis. Early events during the histogenesis of the disease can be identified, and then genotype can be correlated with phenotype using classical techniques in molecular pathology. If one keeps in mind the need to ensure that the animal model recapitulates the pathobiological and molecular features of the human disease, early events in neoplastic progression can often be studied that are difficult if not impossible to derive from human surgical and autopsy material. Renal tubular epithelial carcinogenesis is a disease for which there is a need to use animal models to study early events because these stages are not recognized clinically in humans. Very early preneoplastic stages in tubular transformation in hereditary RCC can be identified in Eker rats [3]. Our laboratory has shown that the overexpression of TGF-α, which is known to occur in RCC, also occurs in preneoplastic lesions. Recently, laser microdissection was utilized to extend the finding of loss of heterozygosity in the region of the *Tsc2* locus on rat chromosome 10 in Eker RCC to preneoplastic renal tubular lesions [11].

In addition to providing a method by which to correlate specific cellular and molecular alterations with early phenotype, studies of the histogenesis of tumors in Eker rats should provide clues regarding the development of renal cell tumors within different parts of the nephron. Considerable controversy exists about the site of origin of RCC within the nephron. Determination of the pathogenesis of these lesions will aid the experimental pathologist and toxicologist in understanding cell type-specific susceptibility to chemical carcinogens. This type of information will aid in cancer modeling for risk assessment by defining the cell populations susceptible for transformation. For example, Eker rat renal tumors exhibit markers of both proximal and distal nephron [12]. Whether this dual marker expression is due to dedifferentiation of transformed cells or if both parts of the nephron are subject to *Tsc2*-associated transformation is not known.

3. In vitro studies with Eker rat renal cells and tumor-derived cell lines

One useful attribute of animal models is that cell lines from tumors of interest can often be established. A panel of tumor-derived cell lines has been established from Eker rat RCC and has proven useful for cytogenetic studies. These cytogenetic experiments have demonstrated that specific alterations occur in rat chromosomes 4, 5, and 6 consisting of loss of all or portions of these chromosomes [13]. It is likely that molecularly dissecting these chromosomal regions will lead to the identification of additional genes important in RCC development. Studies with tumor-derived cell lines from animal models allow one to study potentially important genetic alterations that can then be examined in primary tumor material. In this way, one can determine which genetic events are primary events in the tumorigenesis versus those that are associated with the establishment of tumor-derived cell lines. For example, recent studies with tumor-derived cell lines from Eker rat RCC suggested that involvement of $p15^{ink4B}$ and $p16^{ink4}$ is associated with the acquisition of autonomous growth capacity in vitro and does not appear to be involved in the primary tumors [14].

Animal carcinogenesis models often allow one to develop in vivo and in vitro approaches in which to study phenomena associated with the transformation process. This has been the case for the Eker RCC model. An in vitro rat kidney epithelial (RKE) transformation assay for normal rat kidney cells was established [15]. In this assay, a single cell suspension is obtained from a normal rat kidney and is plated into tissue culture at clonal density. In the absence of carcinogen, normal cells proliferate for a short period of time but then senesce and die, sloughing off the dish. When cells are treated with a carcinogen, however, foci of morphologically transformed cells appear in the dishes at 6–8 weeks with a frequency proportional to the dose

of the carcinogen. Transformed foci give rise to immortal cell lines with tumorigenic potential.

This in vitro RKE transformation assay was adapted for use in studying mechanisms of transformation in the Eker rat model. Kidney epithelial cells from rats heterozygous for the Eker mutation were compared to kidney epithelial cells derived from wild-type rats for their in vitro susceptibility to transformation by the direct-acting carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The percent transformation frequency following MNNG treatment was 7.5-fold higher in cells derived from carrier animals when compared to cells from noncarrier animals [16]. This increased susceptibility to transformation exhibited by the Eker rat cells is consistent with a predisposition resulting from inactivation of the *Tsc2* tumor suppressor gene. The increased susceptibility of kidney epithelial cells carrying the Eker mutation may prove useful in the further development of the RKE transformation assay as a sensitive tool for toxicologists who wish to screen chemicals as potential renal carcinogens.

4. Carcinogenesis studies in Eker rats

In addition to a familial predisposition to tumor development, Eker rats are highly susceptible to the effects of chemical and physical carcinogens. In early experiments conducted prior to the identification of the Eker gene, rats heterozygous for the Eker mutation that were exposed to external radiation had an 11–12-fold enhancement in tumor multiplicity, with a linear dose-response relationship [17]. These experiments clearly implied that only 1 additional genetic event was necessary, consistent with the 2-hit hypothesis. In contrast, studies in normal rats demonstrated a quadratic dose-response relationship to radiation, indicating that 2 or more events are needed in the absence of a predisposing mutation.

Studies in our laboratory with the genotoxic chemical carcinogen dimethylnitrosamine (DMN) showed similar results to those noted with radiation-treated rats heterozygous for the Eker mutation. Carrier Eker rats were treated with DMN, which is known to target both renal mesenchymal and renal tubular epithelial cells. With tumor multiplicity as an end point, there was a 70-fold increase in renal epithelial tumor susceptibility in male heterozygote Eker carriers exposed to DMN but no increase in susceptibility to renal mesenchymal tumors [18]. The combined effect of the Eker mutation and carcinogen exposure resulted in an increase of 3 to 4 orders of magnitude in tumors relative to the spontaneous RCC frequency in wild-type rats. The effects of the Eker germ-line mutation on cancer susceptibility were cell type specific, predisposing to tumor development in epithelial but not mesenchymal cells of the kidney, even in the face of a potent genotoxic carcinogen.

Hino et al. demonstrated that a transplacental regimen of ethyl nitrosourea (ENU) leads to very early induction of renal tubular epithelial tumors in rats carrying the Eker mutation [19]. This transplacental treatment with carcinogen does not lead to the induction of nephroblastomas as one might expect. This provides an additional example of a situation where Eker rats treated with a potent genotoxic chemical carcinogen, known to target multiple cell types, developed only renal tubular epithelial tumors in increased frequency. The *Tsc2* mutation therefore appears to confer exquisite cell specificity to increased cancer susceptibility following carcinogen exposure.

5. Utility of Eker rat cancer model for toxicologists

The kidney is an important target site for the development of tumor responses in rats used in chronic bioassays of toxicity and carcinogenicity bioassays. It is the second most frequent site of transformation in male rats used by the National Toxicology Program in chronic rodent bioassays. Thus determination of the mechanisms of chemically induced rat renal cell tumors is important and can be facilitated by having a high-incidence, spontaneous model that develops tumors with short latency.

There is a great need to establish the relationship between chronic wounding of the kidney by nephrotoxicants and the development of renal tumors. The Eker rat is presently being used to

examine the role of renal cell proliferation induced by cytotoxic and mitogenic agents in the genesis of RCC. Studies are also underway in our laboratory to determine the effects of hormonal status on cell proliferation and renal tumorigenesis. We anticipate that data from these experiments can be applied to carcinogenesis models for refinement of chemical risk assessments. There is presently a particular need for examining the role of nongenotoxic chemicals in renal tumor promotion. The Eker rat model offers a potential means to study this issue because short-latency lesions of various stages in tumorigenesis can be reproducibly established in animals, which can be treated with compounds of interest.

Most importantly, it is becoming increasingly obvious that cancer risk is not equally distributed throughout the human population, and that there are important genetic predispositions that need to be considered in cancer risk assessment. Meaningful mechanistically based cancer risk assessments will ultimately require an understanding of the comparative pathways of molecular carcinogenesis in rodents and humans. Studies of renal cancer in Eker rats should provide the means by which these pathways can be compared for RCC, and may serve as a model for other tumor types.

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Genetic susceptibility and carcinogen-DNA adduct formation in human urinary bladder carcinogenesis[1]

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Abstract

Differences in human urinary bladder cancer susceptibility have often been attributed to genetic polymorphisms in carcinogen-metabolizing enzymes, especially those involved in the biotransformation of aromatic amines (AAs) and polycyclic aromatic hydrocarbons (PAHs). Metabolic activation generally involves an initial cytochrome P450-dependent oxidation to form *N*-hydroxy, phenol, or dihydrodiol intermediates that undergo further conjugation or oxidation to form DNA adducts. The acetyltransferases, NAT1 and NAT2, can participate in these pathways by catalyzing detoxification (by AA *N*-acetylation) or further activation (by N-OH-AA *O*-acetylation) reactions. NAT2 polymorphisms, which are due to point mutations in the structural gene, have long been associated with higher risk for bladder cancer. In collaborative studies, we now have found that NAT1 is also expressed polymorphically in human bladder due to mutations in the NAT1 polyadenylation signal, which has recently been associated with increased bladder cancer risk. Moreover, we have found that the bladder NAT1*10 genotype and phenotype are correlated with significantly higher levels of putative AA-DNA adducts in human bladder as measured by ^{32}P-postlabelling. Preliminary data have also suggested that putative PAH-DNA adducts in human bladder are correlated with a polymorphism in the total metabolism of benzo[*a*]pyrene (BP) by bladder microsomes and especially with the formation of BP-7,8-diol. Since each of these correlations was observed without adjusting for carcinogen intake, it would appear that, with ubiquitous human exposure to AAs and PAHs, the expression of carcinogen-metabolizing enzymes may be a more critical determinant of carcinogen-DNA adduct formation and of individual cancer susceptibility.

Keywords: Aromatic amines; Polycyclic aromatic hydrocarbons; Urinary bladder; Acetyltransferases; Polymorphisms; DNA adducts

1. Enzyme polymorphisms, DNA adducts, and cancer susceptibility

The metabolic activation and detoxification pathways associated with the carcinogenic aromatic amines (AAs) and polycyclic aromatic hydrocarbons (PAHs) provide a useful model of enzyme polymorphisms that can modulate human carcinogenesis. Such interindividual variations in AA- and PAH-metabolizing enzymes can be due to genetic factors or to sustained interaction with environmental factors, either of which can be shown to be a determinant of

* Corresponding author. The genotyping portion of these studies were carried out in collaboration with Drs. Douglas A. Bell and Ari Hirvonen at the National Institute for Environmental Health Sciences, Research Triangle Park, NC and are described in full in Refs. [17–19].

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cancer risk in epidemiological studies [1]. Our efforts have been focused on the risk factors associated with human urinary bladder carcinogenesis and the enzymatic systems that are known to play a major role in the biotransformation of carcinogenic AAs and PAHs such as the *N*-acetyltransferases (NATs) and the cytochromes P450 (CYPs). Metabolic polymorphisms in these enzymatic systems would thus be expected to affect the levels of DNA adducts in the carcinogen-target tissues and thus modulate bladder cancer risk [2].

For AA-induced urinary bladder carcinogenesis (Fig. 1), current hypotheses [3–5] indicate that most AAs are initially metabolized in the liver through either *N*-hydroxylation by CYP1A2 or *N*-acetylation by NAT2. The *N*-hydroxy metabolite can be further metabolized in the liver by sulfotransferases and glucuronyltransferases to form phenolic sulfates and *N*-glucuronides that are major excretion products; or it can enter the circulation where it can be oxidized to a nitrosoarene that forms covalent adducts with hemoglobin. The remaining *N*-hydroxy metabolite then undergoes renal filtration into the urinary bladder lumen where it can be reabsorbed into the bladder mucosa. Although *N*-hydroxy arylamines can react with DNA at acidic urinary pH, further activation by NATs in the bladder has been suggested as a final activation step leading to DNA adducts, mutations and neoplasia. Enzyme polymorphisms that have been previously associated with these pathways include CYP1A2 and NAT2 [6]. Accordingly, the slow NAT2 phenotype, which arises as a consequence of point mutations in an intron-less gene, has long been associated with increased bladder cancer risk [7]; and more recently, cigarette smokers who are slow NAT2 and rapid CYP1A2 were found to possess the highest level of AA-hemoglobin adducts [8].

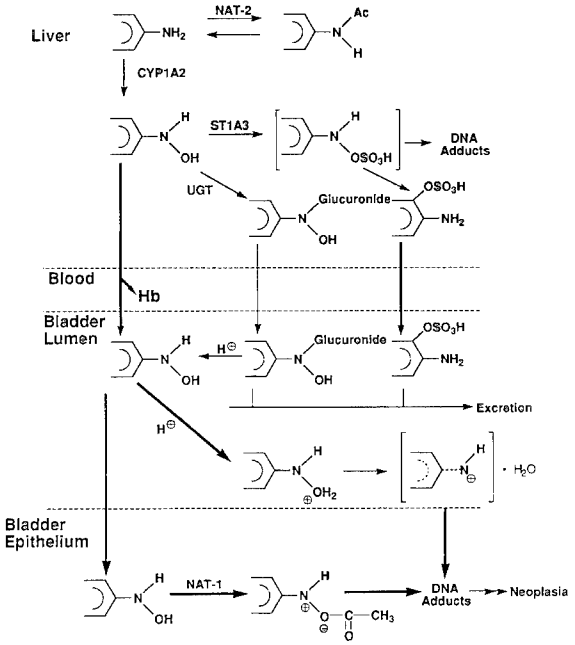


Fig. 1. Proposed metabolic activation and detoxification pathways for AA-induced human urinary bladder carcinogenesis.

In comparison to the AAs, the activation and detoxification pathways for PAHs in relation to urinary bladder carcinogenesis are less clear. However, benzo[*a*]pyrene (BP) is metabolized by cultured human bladder systems [9] and its metabolism in other human tissues such as lung and larnyx is consistent with the initial formation of BP-7,8-diol and 9-hydroxy-BP and their subsequent conversion to DNA-reactive bay-region diol-epoxides and K-region oxides (Fig. 2). CYPs 1A1, 2C9, and 3A4 have each been shown to catalyze these oxidative reactions in human tissues (reviewed in [10]); while glutathione *S*-transferases (GSTs) serve an important role in the detoxification of the arene oxides. In this regard, individuals lacking the GST M1 gene have been associated with a significantly increased risk to bladder cancer [11].

Carcinogen-DNA adducts have been detected in human urinary bladder using ^{32}P-postlabelling methods (butanol extraction and nuclease P1 enhancement) that are selective for AA- and PAH-DNA adducts, respectively [12]. Current cigarette smokers had 2–3-fold higher adduct levels; and nearly half of the adducts detected were similar to those derived from PAHs, while the remainder exhibited properties consistent with their identity as AAs. Of these, the C8-dG adduct of 4-aminobiphenyl (ABP), which accounted for about 10% of the smoking-related adducts, was specifically identified and its pres-

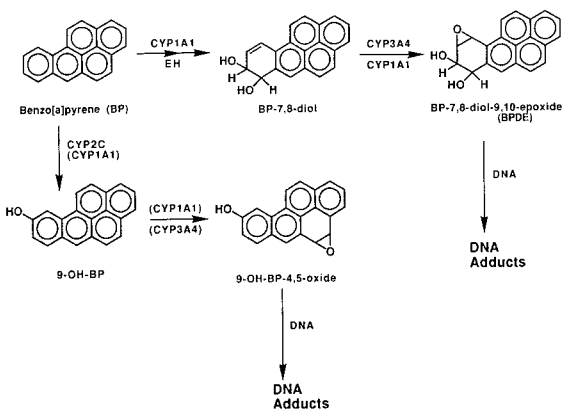


Fig. 2. Potential pathways for the metabolic activation of BP and other PAHs in human tissues.

ence in human bladder DNA was subsequently confirmed by GC/MS [13].

2. Metabolism of AAs and PAHs by human urinary bladder tissues and its role in carcinogen-DNA formation

To assess further the role of AAs and PAHs in human urinary bladder carcinogenesis, we have recently examined the relationship between metabolism and DNA adduct levels in 26 human bladder tissue samples obtained through the US Cooperative Tissue Network. Patient history and exposure information was not available for these samples, although historically some 80–90% of such samples originated from current cigarette smokers. The metabolic *N*-acetylation of *p*-aminobenzoic acid (PABA) to *N*-acetyl-PABA (NAT1 activity) and of sulfamethazine (SMZ) to *N*-acetyl-SMZ (NAT2 activity), and the *O*-acetylation of *N*-hydroxy-ABP (OAT activity; catalyzed by NAT1 and NAT2) was measured in bladder cytosols; and the capacity of bladder microsomes to catalyze oxidative metabolism of BP was determined. DNA was also isolated for determination of *NAT1* and *NAT2* genotype and for analyses of carcinogen-DNA adducts using the butanol extraction and nuclease P1 enhancement methods.

Substantial levels of both PABA and OAT activities were found in all of the bladder cytosols (Table 1). However, SMZ activities were below the assay limits of detection. This observation confirmed the findings that NAT2 activity is poorly expressed in human bladder cytosols in relation to NAT1 activity [14]. In addition, nearly all of the bladder microsomes metabolized BP at low but readily detectable levels (Table 1).

^{32}P-Postlabelling analysis for putative car-

Table 1
Metabolism and DNA adduct levels (RAL) in human urinary bladder

Measurement (units; number of individuals)		Mean ± S.D.
(A)	PABA *N*-acetylation (nmol/min/mg protein; $n = 26$)	2.9 ± 2.3
	SMZ *N*-acetylation (pmol/min/mg protein; $n = 26$)	<10
	N-Hydroxy-ABP OAT (pmol bound/mg DNA; $n = 26$)	43 ± 21
	Total BP metabolism (pmol/min/mg protein; $n = 22$)	1.95 ± 0.62
	RAL (AA-DNA adducts/10^8 dNp; $n = 22$)	2.4 ± 2.0
	RAL (PAH-DNA adducts/10^8 dNp; $n = 22$)	2.5 ± 2.1
(B)	RAL (AA adducts/10^8 dNp); rapid NAT1 phenotype ($n = 13$)	1.7 ± 1.7*
	RAL (AA adducts/10^8 dNp); slow NAT1 phenotype ($n = 13$)	3.2 ± 2.2*
	RAL (PAH adducts/10^8 dNp); rapid BP metabolizer ($n = 11$)	1.6 ± 1.1**
	RAL (PAH adducts/10^8 dNp); slow BP metabolizer ($n = 11$)	3.6 ± 2.2**
(C)	PABA *N*-acetylation (nmol/min/mg protein); *NAT1*4/NAT1*4* genotype ($n = 17$)	2.3 ± 2.6***
	PABA *N*-acetylation (nmol/min/mg protein); *NAT1*4/NAT1*10* genotype ($n = 8$)	4.6 ± 1.6***
	RAL (AA adducts/10^8 dNp); *NAT1*4/NAT1*4* genotype ($n = 17$)	1.8 ± 1.9****
	RAL (AA adducts/10^8 dNp); *NAT1*4/NAT1*10* genotype ($n = 8$)	3.5 ± 2.1****

* $P = 0.05$; ** $P = 0.02$; *** $P = 0.03$; **** $P = 0.05$, using the Mann-Whitney rank sum test.

cinogen-DNA adducts gave distinct chromatographic profiles that were indicative of both AA- and PAH-DNA adducts and at similar levels (Table 1A). Furthermore, the AA-DNA adduct levels in these tissues correlated with their NAT1-dependent PABA activities; and the PAH-DNA adducts correlated with both the total BP-metabolizing capacity as well as with the formation of BP-7,8-diol, a known proximate carcinogenic metabolite (Table 2).

Statistical analyses and probit and NTV plots indicated that both NAT1 activity and total BP metabolism in the human bladder tissue samples were not normally distributed and appeared bimodal. For NAT1 and BP activities, these observations allowed arbitrary designation of slow and rapid (2-fold higher) acetylation phenotypes, with cutpoints near their median values. Within each of these subgroups, NAT1 correlated well with OAT, which provided additional support for the existence of 2 NAT1 phenotypes since this correlation was unobtainable when all NAT1 and OAT data points were examined together. Moreover, the apparent polymorphisms both in NAT1 and in BP metabolism were associated with significant differences in the levels of the corresponding DNA adducts in the bladder tissues examined (Table 1B). Specifically, the rapid NAT1 and rapid BP metabolizer phenotypes each exhibited a 2–3-fold higher level of putative AA- and PAH-DNA adducts, respectively. Thus, even in the absence of exposure information in these individuals, DNA adduct levels in the human urinary bladder appear to be influenced predominantly by individual differences in bladder NAT1- or BP-metabolizing activity.

3. A genetic polymorphism in *NAT1* and its relation to NAT1 phenotype and AA-DNA adduct levels in human urinary bladder

NAT1, which codes for the acetyltransferase activity originally thought to be monomorphic, has recently been shown to exhibit polymorphisms in non-coding regions [15]. The predominant alleles, designated NAT1*4 and *NAT1*10*, differ by a single base at nt 1088, which represents an alteration in the consensus polyadenylation signal. Examination of DNA sequence polymorphisms in the *NAT1* gene by PCR have demonstrated that the *NAT1* polyadenylation polymorphism is associated with significant differences in bladder NAT1 enzyme activity (Table 1C). Accordingly, NAT1 activity in the bladder of individuals with the heterozygous *NAT1*10* allele was 2-fold higher than in subjects with the homozygous *NAT1*4* allele. Furthermore, putative AA-DNA adduct levels in the urinary bladder were similarly found to be 2-fold higher in individuals with the heterozygous *NAT1*10* allele, as compared to those with *NAT1*4* allele. Thus, these data provide strong support for the hypothesis that NAT1 activity in the urinary bladder mucosa represents a major

Table 2
Correlation coefficients (r) and P values

Comparisons	r (P)
NAT1 activity vs. RAL (AA-DNA adducts)	0.52 (0.01)
OAT activity vs. RAL (AA-DNA Adducts)	ns[a]
Total BP metabolism vs. RAL (PAH-DNA adducts)	0.52 (0.02)
BP-7,8-diol formation vs. RAL (PAH-DNA adducts)	0.71 (0.02)
9-Hydroxy-BP formation vs. RAL (PAH-DNA adducts)	0.44 (0.06)
NAT1 activity vs. OAT	ns[a]
Slow NAT1 phenotype vs. OAT	0.66 (0.01)
Rapid NAT1 phenotype vs. OAT	0.71 (0.01)

Correlation coefficients and P values were calculated using the Spearman rank correlation test.
[a] ns, not significant.

bioactivation step that converts urinary *N*-hydroxy arylamines to reactive *N*-acetoxy esters that form covalent DNA adducts (Fig. 1).

Since previous studies have indicated that hepatic NAT2 activity is an important detoxification step for bladder carcinogenesis and individuals with slow *NAT2* genotype were found to be at higher risk for bladder cancer [16], one would predict that individuals who possess a slow *NAT2* and the rapid *NAT1*10* genotypes would be at highest risk of developing this type of neoplasm. Indeed, this combined genotype (slow *NAT2/NAT1*10*) indeed exhibited the highest carcinogen-DNA adduct level in our study. Moreover, slow NAT2 phenotype and rapid CYP1A2 have been implicated in the activation (*N*-oxidation) and detoxification (*N*-acetylation) of AAs for human bladder carcinogenesis.

In conclusion, these data provide the first evidence for phenotypic and genotypic polymorphisms in both NAT1 and NAT2 and a phenotypic polymorphism in the metabolic activation of BP that are predictive of DNA adduct levels in the human urinary bladder. Moreover, the enzyme which has long been thought to be responsible for monomorphic NAT1 activity, is shown to be regulated by a polymorphic gene, *NAT1*, and it is bimodally distributed in the human urinary bladder in a manner that is significantly correlated with the DNA adduct levels found in this tissue. Together with the known polymorphisms for NAT2, CYP1A1, CYP1A2, GSTM1 and sulfotransferase, it is evident that metabolic phenotypes/genotypes can significantly influence DNA adduct formation in urothelial cells and could ultimately lead to a wide variation in urinary bladder cancer risk. Therefore, future epidemiological studies will need to include molecular biomarkers of individual susceptibility, especially those involved in carcinogen activation and detoxification.

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Xenobiotic metabolism in brain

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Abstract

Recent hypothesis suggesting a role for environmental toxins in the pathogenesis of neurodegenerative disorders has stimulated interest in research on xenobiotic metabolizing capability of the brain. In addition to possible irreversible loss of neurons through bioactivation in situ in the nervous tissue, the metabolism of psychoactive drugs in the target tissue can lead to local pharmacological modulation at the site of action. The major drug metabolizing enzymes, cytochromes P-450 (P450) and flavin-containing monooxygenase (FMO) have been detected in rodent brain and human brain tissue obtained at autopsy. The brain microsomal and mitochondrial P450 systems are capable of metabolizing a variety of xenobiotics, while the brain FMO efficiently metabolizes a variety of psychoactive drugs to their respective *N*-oxides. Immunocytochemical studies have revealed the regional heterogeneity in the distribution of multiple forms of P450 in the brain and the co-localization of P450 and FMO predominantly in the neuronal cells. Although the brain P450 and FMO share many common features with similar enzymes present in other tissues such as liver and lung, there are some distinctive differences. It is evident from the studies carried out so far that the brain can metabolize a variety of lipophilic xenobiotics that enter by way of the blood stream.

Keywords: Brain; Cytochrome P-450; Flavin-containing monooxygenase; Drug metabolism; Monooxygenase

1. Introduction

The brain is the most sensitive and complicated organ of the human body; it is the seat of intelligence, interpreter of senses and controller of movement. The complexity of the brain extends to its distinctive anatomical and cellular features. Brain exhibits tremendous regional heterogeneity and each region displays specialized and selective functions. In addition to the regional and cellular heterogeneity, the brain possesses several extraordinary features. The brain is protected from the blood-borne chemicals by the tight junctions present between the endothelial cells forming the blood-brain barrier, with the exception of the circumventricular organs, which have a neuroendocrine function. In addition, the choroid plexus (the major site of cerebrospinal fluid (CSF) production) also restricts the entry of many substances forming a blood-CSF barrier. However, certain relatively more lipophilic xenobiotics can diffuse through

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the endothelial cells of the brain capillaries and thus penetrate into the brain.

The brain is particularly vulnerable to damage by toxic compounds due to the limited regenerative capability of the neuron, which is primarily involved in specialized functions of the nervous system. The cellular toxicity of such compounds, occurring either directly or after in situ metabolism, can have far-reaching consequences by causing irreversible disruption of neuronal function. A role for environmental toxins in the etiopathogenesis of certain neurodegenerative disorders has also been suggested [1]. In addition to being a potential locus of toxicologically significant in situ metabolism of certain compounds, neuronal cells may similarly be a locus of pharmacologically significant metabolism of certain therapeutic agents. Thus, metabolism of certain psychoactive drugs directly in the brain may lead to local pharmacological modulation at the site of action and result in variable drug response. Generally, phase I metabolism (mediated by microsomal monooxygenases, cytochrome P-450 (P450) or flavin-containing monooxygenases (FMO)) results in the formation of hydrophilic metabolites that are removed by renal clearance. However, in the brain, the formation of such metabolites would result in prolonging the half-life of the metabolite due to the presence of the CSF-blood barrier.

2. Rat brain P450

The first attempt at quantitation of the heme protein P450 in rat brain from the reduced carbon monoxide binding spectrum was reported by Sasame et al. [2] and later by Marietta et al. [3]. The reported brain P450 levels are 25–40 pmol/mg microsomal protein or approximately 2.5–4% of the specific contents of the hepatic tissue [4]. Mitochondrial P450 content in brain is significantly higher than the microsomal P450 content, in contrast to the liver [5,6].

The quantitative measurement of P450 in brain has been difficult due to (i) interference from other chromophores and (ii) the turbidity caused by large amounts of protein needed to measure the low levels of P450 present in brain. In our experience transcardial perfusion of the brain prior to decapitation followed by preparation of the microsome in presence of dithiothreitol (0.1 mM), EDTA (0.2 mM), phenylmethyl sulfonyl fluoride (0.1 mM) in tris-potassium chloride buffer containing 10–20% glycerol (previously bubbled with nitrogen) results in a preparation that contains comparatively higher amounts of P450 [7]. The reduced carbon monoxide spectrum of brain microsome from untreated rats prepared in this fashion has an absorption maximum at 452 nm and is amenable to quantitative measurement of P450. The brain microsomal P450 content has been determined to be 80–100 pmol/mg protein (Fig. 1). The reduced carbon monoxide spectrum of brain microsomes prepared in the conventional manner has a small deflection at 450 nm and the quantitation of P450 from the spectrum is difficult. It thus seems important to adequately preserve the integrity of the heme protein during preparation of the microsomes.

Several P450-mediated monooxygenase activities are detectable in brain microsomes. The ethoxycoumarin *O*-deethylase activity in brain microsomes prepared in the presence of dithiothreitol and glycerol are typically 20% of the hepatic activity in untreated rats [8]. *N*-Nitrosodimethylamine *N*-demethylase activity is also detectable in brain [9]. The bioactivation of this

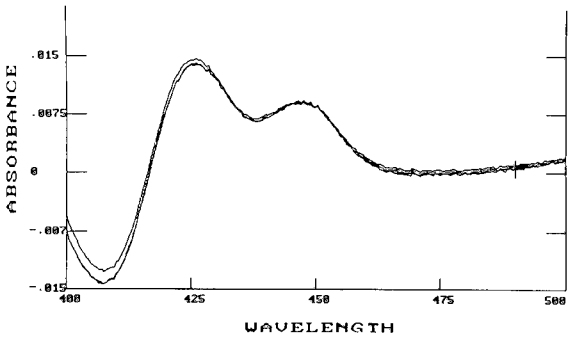


Fig. 1. The dithionite reduced carbon monoxide difference spectrum of rat brain microsomes. The protein concentration was 1 mg/ml. The specific content of P450 was estimated to be 100 pmol/mg protein.

potent carcinogen in situ in the brain could lead to neoplastic transformation.

Tremendous variations are observed in the reported values of the specific activities of the P450-associated monooxygenase in brain. The different methods used for the preparation of the microsomes could possibly explain some of these differences, as only a fraction of the total P450 is recoverable in the brain microsomes prepared by conventional methods. Further, linear dependence of monooxygenase activity with increasing enzyme concentration is observed only over short ranges of microsomal protein concentration and in general multiphasic kinetics are observed over a broad range of enzyme concentration. This has been attributed to the change in the ratio of enzyme to substrate concentration that occurs while carrying out reactions over a wide range of enzyme concentration [7]. Thus, when morphine *N*-demethylase activity was determined with 7 μg brain microsomal protein/ml incubation medium containing 1.8 mM morphine wherein the ratio of microsomal protein (in μg/ml) to substrate concentration (in μmol/ml) is 3.89, the activity was determined to be 127 $\pm$ 19 nmol formaldehyde formed/min/mg protein. Under the same conditions, when 70 μg microsomal protein was used for the assay (the ratio of enzyme to substrate = 38.9), the measured activity was only 30 nmol formaldehyde formed/min/mg protein. However, when the substrate concentration in the incubation medium was increased such that the above ratio was maintained at 3.89, the microsomal morphine *N*-demethylase activity was determined to be 92 $\pm$ 4.5 nmol formaldehyde formed/min/mg protein, which was similar to that seen when 7 μg protein was used for the assay [7]. Thus, the amount of microsomal protein in the incubation medium and, more importantly, the ratio of enzyme to the substrate concentration are major determinants of the specific activity measured. The amount of microsomal protein used in the incubation for measurement of monooxygenase activities has typically varied from 10–20 μg/ml [8] to 15 mg/ml [3]. In addition, the incubation time for measurement of monooxygenase activity has varied from 10 to 60 min among various laboratories. Since the rate of product formation is not linear over prolonged periods of time, the activity (expressed as product formed/min) is often underestimated.

The brain exhibits regional differences in the distribution of P450. Maximal P450 levels have been detected in olfactory lobes, cerebellum and brainstem [10]. The presence of high amounts of P450 in the brainstem coupled with the low levels of the endogenous nucleophile, glutathione, known to be present in this region [11] may render the brainstem particularly vulnerable to damage through P450-mediated bioactivation of xenobiotics to reactive electrophilic metabolites.

Multiple forms of P450 are constitutively expressed in brain and share immunological similarity with the corresponding, well-characterized hepatic forms such as P450 2B1/2B2, 1A1, 1A2 and 2E1. The isoforms of brain P450 are selectively inducible by phenobarbital, 3-methylcholanthrene [8] and ethanol [9]. Regional variation has been observed in the induction of P450 by phenobarbital and ethanol. Two-fold induction in the P450 levels were observed in the cortex, brainstem and thalamus, following administration of phenobarbital. However, the P450 levels in the cerebellum and hippocampus were unaltered. In contrast, when rats were administered ethanol for 1 month, maximal P450 induction was seen in striatum, hippocampus and thalamus, while the P450 levels in cortex, cerebellum and brainstem were unaffected. These observations indicate the differential distribution and regulation of various isoforms of P450 in distinct brain regions. Interestingly, administration of nicotine results in selective induction of brain P450 and certain monooxygenase activities, while having no effect on liver P450 [10]. This apparently selective effect of nicotine on brain P450 is of significance as it indicates the possible regulation of brain P450 by mechanisms independent of and different from those known for hepatic P450s. Further, the induction of P450 and selective monooxygenase activities in specific brain regions following administration of phenobarbital (a commonly used anticonvulsant drug), ethanol or nicotine indicates the possibility of increased in situ bioactivation of xenobiotics

and/or alteration in the pharmacodynamics of drugs acting on the central nervous system. The induction of *N*-nitrosodimethylamine *N*-demethylase activity in rat brain following ethanol administration suggests the possibility of increased bioactivation of this potent carcinogen in brain following alcohol intake. The induction of P450 in certain brain areas following administration of phenobarbital is of significance, since this anticonvulsant drug is often administered chronically to patients and combination drug therapy in such patients could result in altered pharmacological response.

In addition to the microsomal P450, the mitochondrial P450 in the brain is also involved in the metabolism of xenobiotics. The P450 level in brain mitochondria is twice that of the corresponding microsomal concentration and certain monooxygenase activities in the brain mitochondria (such as *N*-nitrosodimethylamine *N*-demethylase) are significantly higher as compared to microsomes [5]. Multiple forms of P450, which are selectively inducible by ethanol and phenobarbital are detectable in brain mitochondria and these isoforms of P450 share immunological similarity with the corresponding microsomal forms as evidenced by immunoblot and immunoinhibition experiments. Thus, the total P450 and associated monooxygenase in brain microsomes and mitochondria needs to be taken into account when considering the xenobiotic metabolizing capability of the brain.

The immunohistochemical localization of P450 in untreated rat brain has been performed using the antiserum to purified phenobarbital-inducible form of brain P450. Since the antiserum used recognizes both the mitochondrial and microsomal P450, the immunocytochemistry reflects the localization of both the microsomal and mitochondrial P450 in the brain. The immunostaining of P450 was essentially localized in phylogenetically older brain parts like olfactory area, pyriform cortex, cingulate cortex, brainstem and cervical cord. The neuropil staining was evident in the various neuroanatomical areas. In most cells the staining was perikaryal. The white matter was essentially excluded from the immunostaining. The fibre tracts stood in light contrast against the background of neuropil and neuronal staining. However, the subpial and subependymal glial cells were intensely stained, while the ependymal cell labelling was variable. The vascular elements were not stained.

In addition to the brain, multiple forms of P450 have been detected in the rat spinal cord. The total P450 content in the spinal cord is comparable to that of whole brain. Certain monooxygenase activities (such as aniline hydroxylase, 7-ethoxycoumarin *O*-deethylase and *N*-nitrosodimethylamine *N*-demethylase) were significantly lower in the spinal cord as compared to the brain, indicating that the multiple forms of P450 were differentially distributed between the brain and spinal cord. Immunocytochemical localization studies using the antiserum to rat brain phenobarbital-inducible P450 revealed the localization of the hemeprotein in the anterior horn cells, indicating that these cells may be vulnerable to damage through P450-mediated bioactivation [12]. Amyotrophic lateral sclerosis is a neurodegenerative disorder wherein these neurons are selectively damaged.

3. Human brain P450

The concentration of P450 [13] and associated monooxygenase activities [14] have been demonstrated in microsomes from human brain regions obtained at autopsy. The microsomal P450 levels in the cortex varied between 0.03 to 0.12 nmol/mg protein among the 7 samples examined. The hemeprotein level (expressed as nmol/g tissue) was highest in the brainstem and cerebellum and lowest in striatum and hippocampus, analogous to that seen in rat brain. The distribution of the monooxygenase activities varied among different regions of the brain. The aminopyrine and morphine *N*-demethylase activities were highest in the brainstem region namely, pons, medulla and midbrain. Immunocytochemical localization studies have been carried out using the antibody to rat liver P450 (2B1/2B2) and the immunoreactivity has been shown to be localized in the neuronal cell bodies, especially in the large neurons of the reticular formation and lower

cranial nerve nuclei of the human medulla oblongata. The human brain NADPH cytochrome *c* reductase activity [15] has also been detected in microsomes prepared from human brain tissue obtained at autopsy. The immunohistochemical localization studies using antiserum to purified rat liver NADPH cytochrome P450 reductase has revealed the co-localization of P450 and NADPH cytochrome P450 reductase in the neuronal cells.

These recent studies have demonstrated the presence of integral P450 system in the brain and the need for characterization of the multiple forms of P450 in the brain with a view to determine the possible existence of brain-specific P450 and to ascertain the specific forms of P450 involved in the metabolism of drugs that act on the central nervous system.

The presence of significant amounts of P450 in the brain and their preferential localization in the neuronal cells has raised the important question about the functional role of P450 in the brain. The close association of the dopamine transporter with P4502D in the striatal membrane has raised the possibility that this P450 may be involved in the regulation of the dopamine transporter [16]. The close resemblance between the sigma opiate receptor and P450 has raised a speculative hypothesis that the brain P450 may indeed function as a sigma opiate receptor [17] and evidence supporting and contradicting the hypothesis have been presented.

Our laboratory has also examined the presence of the other major drug metabolizing enzyme FMO in the brain. The enzyme was found to carry out the *N*- and *S*-oxidation of psychoactive drugs and model substrates in both rat and human brain [18,19]. An interesting observation was that brain microsomes exhibited higher affinity (K_m = 16–22 μM) and higher activity (V_{max} = 182–650 nmol NADPH oxidized/min/mg protein) for the metabolism of imipramine and fluoxetine as compared to model substrates such as methimazole (K_m = 600 μM and V_{max} = 31 nmol NADPH oxidized/min/mg protein) and *N*,*N*-dimethylaniline (K_m = 2.8 mM and V_{max} = 340 nmol NADPH oxidized/min/mg protein). More recent studies have revealed that the FMO activity in human brain microsomes measured as the amount of imipramine *N*-oxide formed is significantly higher (2-fold) than the corresponding activity in rat brain microsomes. Immunocytochemical studies have demonstrated the localization of FMO in neuronal cell bodies and established the co-localization of FMO and P450 in brain [18,19].

It is evident from the studies carried out so far that the brain has significant capability to metabolize a variety of xenobiotics including drugs through P450 and FMO, and can therefore efficiently detoxify the lipophilic foreign compounds that enter the brain. However, the presence of significant amounts of P450 in neurons could render these vital cells vulnerable to injury through bioactivation. The histochemical evidence suggesting the selective localization of the endogenous nucleophile glutathione in the glial cells also indicates that neuronal cells may indeed be vulnerable to damage by electrophilic metabolites formed through bioactivation.

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Expression of multiple forms of brain cytochrome P450

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Abstract

Multiple forms of cytochrome P450 (P450) in brain tissue have been demonstrated to be expressible in brain tissue using polymerase chain reaction (PCR) techniques, Northern blotting, hydroxylation activity assessment and cloning approaches. The antidepressant drug imipramine is metabolized by brain microsomes to multiple products by pathways inhibitable by quinidine, 7,8-benzoflavone, and ketoconazole, well-known inhibitors of P450-catalyzed reactions. Moreover, PCR studies revealed that a number of P450s are expressible in brain tissue and in glioma C6 cells. Quantitative PCR studies further demonstrated the response of many of these forms to induction in agreement with hydroxylation activity results.

Keywords: Brain cytochrome P450; Brain drug metabolizing system; Brain P450 isozyme expression

1. Introduction

Cytochrome P450 (P450)-dependent substrate hydroxylation activities have been reported in brain tissue from untreated animals and animals pretreated with xenobiotic inducers or hormones [1–4], pointing to the presence of a drug metabolism system that was solubilized and resolved into its components, cytochrome P450 reductase and P450s by Bergh and Strobel [5]. The purified reductase and partially purified P450 were able to reconstitute substrate hydroxylation activities giving direct evidence for a competent P450-dependent drug metabolizing system in brain.

The range of P450 forms present and expressible in brain has been expanded by purification studies [4–6] and by studies utilizing molecular biological techniques. A wide range of P450 forms was shown to be expressible in brain tissue using polymerase chain reaction (PCR) [7]. Schilter and Omiecinski [8] used Southern blot techniques to show the distribution of some P450s in various brain regions. Our laboratory has been interested in the function and distribution of P450s in brain [9]. We have studied the distribution P450s 2D in brain and their response to xenobiotic inducers and hormones [10]. Here we summarize evidence for the function of a P450 2D form or forms in brain microsomes and describe the isolation from a brain cDNA library of a form of P450 2D which seems to be uniquely expressed in brain tissue [11]. We also report here the activity of P450 forms in glioma cells [12,13].

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2. Materials and methods

The methods of approach and materials utilized are as previously reported [5,7,9–13]. Salient features of the experiments are described in the Results and discussion section while the particular details are contained within the references cited.

3. Results and discussion

3.1. Hydroxylation activity in brain tissue microsomes

While it has been generally accepted that the liver is the organ most active in drug metabolism roles, other organs have come to the fore in discussions of the metabolic fate of particular drugs. The activation of a particular carcinogen in a specific organ perhaps catalyzed by a specific P450 could initiate a process eventuating in tumor formation. For instance, the activation of dimethylhydrazine in colonic tissue may play a role in the development of tumors of the colon following exposure to the colon-specific model carcinogen 1,2-dimethylhydrazine or its naturally occuring precursor cycasin [14]. Likewise, the presence of particular P450s in brain tissue may direct the metabolism of drugs in brain toward particular product profiles. While this may be generally true for substrates in brain, it is of particular importance for those drugs which act in the brain to treat specific brain dysfunctions. Since the brain plays so great a role in regulating many other body processes, alteration in drug metabolite profiles may have as great an effect on the regulation of processes in distantly located organs as it does in the brain itself.

We have investigated the metabolism of the tricyclic amine antidepressant imipramine by brain microsomes. The metabolism of imipramine is linear with time for 40 min and linear with microsomal protein concentration up to 2.5 mg protein (data not shown). More than one metabolite is produced [9]. The effects of known P450 inhibitors on 2 imipramine metabolites are shown in Table 1. Ketoconazole and 7,8-benzo[*a*]flavone each inhibit several forms of P450 including 1A1, 1A2, 2A1, 2E1 and 3A. These 2 inhibitors have roughly the same degree of inhibition of the formation of hydroxy imipramine products. Ketoconazole, however, is 3 times as effective in inhibiting desipramine formation than is 7,8-benzo[*a*]flavone. Quinidine shows a quite different pattern in that it inhibits very strongly the formation of hydroxylated products but does not affect demethylation. The effects of these 3 inhibitors suggest that more than one P450 is involved in the metabolism of imipramine. The effects of quinidine strongly suggest the involvement of a 2D family member in the formation of hydroxy imipramine products but not in demethylated products.

3.2. Identification of P450 isoforms in brain and glioma C6 cells

The most definitive way to demonstrate the effective presence of a form of P450 includes some form of identification of a form such as purification of the form or immunoblot definition and assay of activity attributable to that form. Clearly a pure form, whether isolated or ex-

Table 1
Effects of inhibitors on hydroxylation and demethylation of imipramine by brain microsomes

Inhibitor added		Percent product formed	
		Hydroxylated imipramine	Desipramine
None		100	100
Ketoconazole	100 μM	58	23
7.8-Benzoflavone	100 μM	50	70
Quinidine	10 μM	42	100
	100 μM	3	92

Table 2
Forms of P450 identified in brain and glioma C6 cells by PCR techniques

Tissue source	P450 forms						
Brain	1A1	1A2	2A1	2B1	2B2	2C7	2C11, 2D, 2E1, 3A
Glioma C6	1A1	1A2	2A1	2B1	2B2	2C7	2 D1/5, 2E1

pressed in an heterologous system, enables the clearest association of an activity with a form. On the other hand, it is also useful to know which P450 forms a tissue can express. In this area the PCR technique is useful. We have examined rat brain RNA for the presence of transcripts for various P450 forms. As summarized in Table 2 a variety of forms can be expressed in brain and even this list is incomplete [6–8,10,11]. There is a remarkable concurrence between what is expressible in whole brain and what is expressible in the model for pure glial cells – the glioma C6 cell line [12,13]. Here also the list is incomplete because the presence of many forms has not yet been examined. The results here indicate only that it is possible to express these forms. The conditions under which any form is normally expressed are still to be defined.

3.3. Effectors of hydroxylation activity in brain and glioma C6 microsomes

The expressibility of forms of P450 as detected by PCR assays and the demonstration of microsomal P450-dependent activities point with clarity to the presence of a regulated P450 system in brain. Since glioma C6 cells show a quite similar pattern of P450 forms expressible, it appears that the P450 system is present in glial cells. To test the regulability of P450 activities in glioma cells, the response of glioma cell microsomal hydroxylation activities following treatment of the cell culture with the known inducers of P450, phenobarbital and benzanthracene, was explored [13]. As summarized in Table 3, similar levels of ethoxyresorufin and pentoxyresorufin hydroxylation activity were detectable in glioma microsomes as in brain microsomes after treatment with benzanthracene or phenobarbital, respectively. In untreated cells, however, neither activity could be detected whereas in untreated brain modest levels of both activities could be demonstrated. Thus for brain and glioma C6 cells the P450 system is regulated by exposure to inducers, as well as other factors. This follows the pattern of induction so clearly demonstrated in liver although the induced levels of activity, though not necessarily the degrees of inducibility, were much higher in liver than in glioma or brain.

3.4. P450 clones isolated from brain cDNA libraries

The ability of brain microsomes to metabolize imipramine and the differential sensitivities of product formation to quinidine and other inhibitors (vide supra) have been further studied by cloning P450 forms from brain cDNA libraries prepared from saline or imipramine-adapted rats [11]. Imipramine-adapted rats were treated daily

Table 3
Hydroxylation activities of brain and glioma C6 cell microsomes

Source	Activity			
	Ethoxyresorufin		Pentoxyresorufin	
	Untreated	BA-treated	Untreated	PB-treated
Brain	0.2	15	0.2	11
Glioma C6	ND	12	ND	9
Liver	24	830	26	729

Activity is expressed in pmol/min/mg microsomal protein.
BA, benzanthracene; PB, phenobarbital; ND, not detectable.

Table 4
P450 clones isolated from a rat brain library

Clone	Total size	Complete	5′ Untranslated end	3′ Untranslated end
2D	1786	Yes	196	90
2D	2754	Yes	194	1060

with imipramine (10 mg/kg) for 3 weeks while saline-treated rats received saline daily for 3 weeks. The libraries were probed with a radiolabelled probe made by random primer labelling the PCR product used previously to identify the 2D subfamily in brain by PCR and quantitative PCR techniques [10]. The probe proved quite useful and sensitive. Table 4 summarizes some characteristics of 2 clones isolated from the imipramine-treated or saline-treated cDNA library. The 2 clones described in Table 4 have identical full-length sequences for the 2D form, but differ in the amount of untranslated sequence at the 5′ and 3′ ends. More sequence is present at the 3′ end than at the 5′ end. As described earlier [11], this clone is quite similar to 2D4 with the exception of 8 base differences in the open reading frame, which give rise to 5 amino acid differences. All of the amino acid differences are located in the C-terminal half of the protein. The homology of the shorter 2D clone with 2D4 is quite high such that when liver and brain are probed with a probe made of the cDNA clone, both liver and brain show positive results, whereas only the brain shows up when the probe used is made to the 3′ untranslated region of the longer clone. These data favor the interpretation that the homology of the clone to 2D4 is such that the probe recognizes 2D4 in the liver and the new clone in the brain. On the other hand, that the 3′ untranslated region probe reacts only with the brain sample and not with the liver sample suggests that the new clones are unique to the brain or at least not expressed in liver. This clone seems to be expressed to an increased level in imipramine-treated rats [11]. The isolation of this clone with high homology to the 2D4 found in liver yet uniquely expressed in brain is consistent with the metabolism results of brain microsomes presented above. In other studies we have isolated 2 forms similar to 3A and 3 unique 4F forms, though the sequencing is not yet completed. These forms extend the list of forms expressed in rat brain and provide a base for the notion that metabolism of drugs, especially drugs active in the brain, may be catalyzed by a unique array of forms present in brain which may respond more readily to stimuli such as imipramine than closely related forms in other tissues.

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Blood-brain interfaces: relevance to cerebral drug metabolism

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Abstract

The brain, with the exception of the circumventricular organs (CVOs), is partially protected from the invasion of blood-borne chemicals by the tight junctions that link adjacent cerebral endothelial cells and form the structural basis of the blood-brain barrier (BBB). In addition to the BBB, the epithelial layer of the choroid plexuses and the barrier layer of the arachnoid membrane complex comprise a second system for protecting the brain, a system often referred to as the blood-cerebrospinal fluid (CSF) barrier. In the past several years, several enzymes that are involved in hepatic drug metabolism have been found in the small microvessels from brain, the choroid plexuses, and the leptomeninges (pia plus arachnoid mater) as well as in some CVOs. These drug-metabolizing systems are inducible and may act at these various interfaces as 'enzymatic barriers' to influx. In particular, the activities of these enzymes in choroidal tissue are so high that the choroid plexuses can well be the major site of drug metabolism in the brain. The fate of intracerebrally formed polar metabolites and the potential of the blood-brain and blood-CSF barriers as sites for metabolic activation-induced neurotoxicity are discussed.

Keywords: Blood-brain barrier; Circumventricular organs; Drug-metabolizing enzymes; Choroid plexuses; Arachnoid membrane

1. Introduction

The cerebral capillaries form the so-called blood-brain barrier (BBB) and are the main interface between the brain and the circulation; a major function of the BBB is the reduction or prevention of blood-borne chemical entry into brain parenchyma [1]. The exchange of toxicologically or pharmacologically active xenobiotics between the brain parenchyma and the cerebral circulation is, however, more complex than that represented by a simple 'two-compartment' model. Additional components of the brain distribution system include blood-cerebrospinal fluid (CSF) exchange across the choroid plexuses and arachnoid barrier layer, CSF-cerebral parenchyma transfer across the ependymal and pial-glial membranes, CSF circulation, and perivascular fluid flow and drainage. Finally, the peculiar brain structures collectively referred to as the circumventricular organs (CVOs) lack a typical BBB [2] and are special sites for drug uptake and neurotoxicity and possibly additional routes of entry into surrounding neural tissue and CSF.

In the last 2 decades, evidence that some xenobiotic metabolism takes place within the brain has been suggested, mostly by detecting and quantifying the activities of several en-

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zymatic complexes known to catalyse the functionalization or the conjugation of exogenous molecules such as drugs and toxic agents present in the environment [3,4]. These enzymatic systems consist of multigene families of isoenzymes and exhibit a broad substrate specificity. Functionalizing enzymes include cytochrome P450s, which catalyse hydroxylation and dealkylation reactions, and epoxide hydrolases, which inactivate reactive epoxides; conjugating enzymes include UDP-glucuronosyltransferases and glutathione *S*-transferases, which, of course, catalyse the conjugation of glucuronic acid and glutathione, respectively, to various substrates.

As the overall metabolic capacities of these enzymes are rather low in the whole brain, the real toxicological significance of these enzymes involves the establishment of their precise structural and cellular localization. For instance, immunohistochemical and transcripts expression studies have shown that the distribution of flavin-containing monooxygenase and of several isoenzymes of cytochrome P450 responsible either for the hydroxylation and dealkylation of various drugs (P450 IIB1,2), alcohol oxidation (P450 IIE1) or polyaromatic hydrocarbon activation (CYP1A family) is heterogeneous among brain structures and among specific populations of neurons and glial cells (e.g., [5–8]). This chapter will concentrate on the localization of some drug-metabolizing enzymes within discrete structures forming the various interfaces between the brain and the extracerebral environment. The potential toxicological relevance of this localization will be discussed together with the potential role of the cerebral circulation pathways in the dissemination and elimination of the metabolites.

2. Blood-brain interfaces and drug transfer into the brain

Some lipophilic compounds may have direct access to brain from the extracerebral environment through the nasal epithelium and along the olfactory nerve into the olfactory lobe [9]. The main pathway of drug entry into the brain is, however, through the general circulation, i.e., by crossing the BBB. The endothelial cells of brain capillaries are joined by continuous belts of tight junctions and are devoid of fenestrae. These tightly linked endothelial cells form the BBB. Because virtually no solute can move through the very restrictive, high resistance intercellular clefts of the cerebral endothelium, xenobiotics will reach most parts of the brain only by passing through the endothelial cell plasma membranes, a greatly limited route of entry for highly hydrophilic molecules. The extent of brain influx for a xenobiotic will, thus, be strongly dependent on its lipophilicity and molecular weight (Table 1) except for molecules that can be carried across the BBB by one of its specific transporter systems. The latter possibility is seldom the case for drugs (melphalan, an anticancer amino acid analog, is an example of a drug whose entry into brain is facilitated by a transporter; see [10]). Other important factors in setting brain influx include protein binding, red cell carriage, cerebral blood flow, and capillary surface area. The latter 2 variables differ among brain regions and are partly responsible for regional differences in drug uptake, disposition, and efficacy [1].

In accordance with the previously described dependency of solute influx into brain, the diffusion coefficient-partition coefficient product of a substance (reflecting the lipophilicity and size of the molecule) is in good agreement with the permeability-surface area product (the physiological expression of capillary permeability) for various 'model' molecules. However, this linkage

Table 1
Factors setting xenobiotic influx across the BBB

- Characteristics of the compound
  - molecular size
  - lipid solubility
  - plasma water concentration, plasma protein and/or red blood cell binding
- Characteristics of the microcirculatory system
  - rate of blood flow
  - surface area available for transport
- Characteristics of the endothelial cells
  - tight junctions
  - transporters
  - multi-drug resistance (P-glycoprotein)
  - drug metabolism

is weaker, more variable, or virtually lacking for many drugs and neurotoxic compounds [11]. This suggests that other processes influence the brain uptake of many drugs. Two of these putative processes are active efflux from brain to the blood by the P-glycoprotein (Pg or P-170), the multidrug resistant protein (MDR) transporter that has been found in cerebral capillaries [12,13], and drug biotransformation within the endothelial cell, which alters both the activity and the lipophilicity of the drug.

The 7 CVOs of the brain are the subcommissural organ, subfornical organ, median eminence, neural lobe of the hypophysis, organum vasculosum of the laminae terminalis (OVLT), pineal gland and area postrema. As their collective name implies, these small brain structures are located in close proximity to the ventricles and, in some cases, also the subarachnoid space. The CVOs, with the exception of the subcommissural organ, have capillaries that lack BBB properties and are surrounded by large fluid-filled perivascular spaces (Fig. 1). The endothelial cells in these 6 CVOs are fenestrated and generally lack tight junctions (the endothelium of the OVLT is sealed by tight junction but is fenestrated). The capillaries in most CVOs are highly permeable to blood borne chemicals such as hormones. This high capillary permeability apparently facilitates the neuroendocrine functions of these tissues (for a review, see [2]). In these structures, the uptake rate of xenobiotics will be mainly set by blood flow and capillary surface area. Both parameters vary greatly among the CVO and are in general equal to or several times greater in CVOs than in cerebral grey matter [14]. Accordingly, these odd brain structures would be expected to be highly sensitive to toxic insults.

The blood-CSF interfaces are represented by the choroid plexuses and the barrier layer of the arachnoid (Fig. 1), the latter constituting a tight membrane between the subarachnoid CSF and the porous vessels of the dura mater. The choroid plexuses lie in the 4 ventricles of the brain. They consist of capillaries that lack BBB properties, a connective tissue-filled interstitium, and a surrounding choroidal epithelium, whose

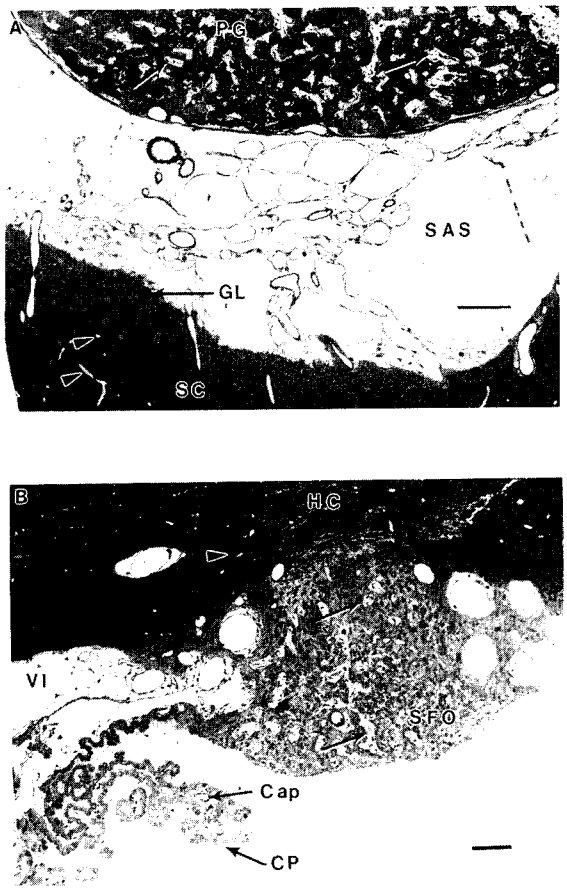


Fig. 1. Light micrograph of rat brain CVOs and adjacent structures. One-micron sections of epon-embedded tissue were stained with toluidine blue. (A) The pineal gland (PG) is surrounded by subarachnoid space and lies on top of the superior colliculus (SC), the latter being covered by a thick glia limitans (GL). (B) The subfornical organ (SFO) lies below the white matter of the hippocampal commissure (HC). Choroid plexuses (CP) can be seen adjacent to the SFO and contain large capillaries (Cap). The velum interpositum (VI) is an extension of the subarachnoid space that ends in the vicinity of the SFO. Note the large perivascular spaces surrounding the capillaries in both CVOs (arrows), whereas the capillaries in the other brain tissue are completely devoid of such spaces (arrowheads) Scale bars represent 100 μm.

cells are joined by tight junctions. In the choroid plexus the barrier is, therefore, shifted from the endothelium to the epithelial covering. The parameters that set brain influx at the BBB (lipid solubility and molecular size) apparently also apply at the 2 blood-CSF barriers. In addition,

the epithelial choroidal cells are known to have special transport systems for ions, amino acids, and other substances. An extensive system of microvilli is present on the apical (CSF-facing) membrane of the epithelial cells, and elaborate interdigitations are seen along their basal surfaces. The choroid plexuses not only secrete CSF but are also involved in neuroendocrine functions, actively deliver micronutrients into the CSF, and 'take-up' metabolites and toxic substances from the CSF. In addition the choroid plexuses are involved in the delivery to and clearance from the CSF of several drugs (e.g., AZT) and of various xenobiotics [15].

3. Potential for drug metabolism in the blood-brain interfaces

The major site of metabolism during drug transfer into the brain by the nasal pathway is probably the non-neural olfactory epithelia. Indeed the nasal cavity contain numerous drug-metabolizing enzymes, some of which are present at very high activities (for a review, see [16]). In the rat and monkey, the olfactory bulbs and olfactory nerve also express some isoforms of cytochrome P450 and UDP-glucuronosyltransferase at higher levels than do brain regions [7,8,17–19].

In general the brain structures lacking a BBB have higher capacities for drug metabolism than the cerebral cortex [20]. In particular the membrane-bound epoxide hydrolase and UDP-glucuronosyltransferase activities are very large in the neural lobe of the hypophysis, and 7-benzoxyresorufin-*O*-deethylase activity, which is an acceptable indicator of cytochrome P450 transformation of xenobiotics, is singularly high in the median eminence.

Differences in the enzyme profiles among the CVOs suggest an endogenous, physiological function for some of these metabolic systems and in some of these particular brain structures. Such a physiological role does not, of course, eliminate the biotransformation of exogenous molecules invading the CVOs by these systems but suggests that there may be some competition for metabolism by endogenous substrates. Nonetheless, such drug-metabolizing systems would provide some enzymatic protection of these fragile brain structures. Finally, the lack of a BBB in the CVOs facilitates the entry into these tissues of molecules that have the ability to induce drug-metabolizing enzymes in the liver and other organs. Such molecules may increase the drug metabolism capacity of the CVOs as has been shown in the pituitary gland [21].

It is likely that neither the xenobiotics nor their metabolites are likely to spread to a significant extent from the CVOs into the adjacent CSF and be carried to other parts of the brain because the membrane separating these peculiar neuroendocrine structures from the CSF appear to be rather tight [2].

The effectiveness of the drug-metabolizing enzyme within the BBB depends on the tight junctions, which seal the intercellular spaces and force materials to pass through the endothelial cells in their course between blood and brain. During this process of passively dissolving and diffusing through the BBB, xenobiotics are exposed to the enzymatic systems of the endothelial cells. The metabolites formed are usually more hydrophilic and are therefore less able to cross the BBB than the parent compounds. There thus may be some problem in eliminating such products from the cells that produce them. On the other hand, these metabolites usually do not have the same pharmacotoxicological properties as the parent molecule and their temporary accumulation may not adversely affect the endothelium. Lastly drugs and related compounds would not be expected to cross the BBB by transcytosis, as seemingly happens with some proteins and peptides, and are thus not likely to escape exposure to the various drug-metabolizing systems.

Cerebral endothelial cells and their immediately adjacent pericytes are ensheathed together within the same basement membrane. In turn this bicellular complex is almost completely covered by astrocytic end-feet or processes. The metabolic capacities of both pericytes and astrocytic processes may play a role in the biotransformation of xenobiotics moving back and forth between cerebral blood and brain parenchyma.

The best known example of a toxicologically

relevant biotransformation of a xenobiotic at the level of the BBB is the metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into its dihydropyridinium form (MPP^+) by mitochondrial monoamine oxidase-B (MAO-B). When present in the brain parenchyma, MPP^+ induces a Parkinson-like syndrome in humans and primates. However, when MPTP is metabolized at the level of the BBB, entry of the resulting MPP^+ into the brain is prevented by its high polarity. Species that have low levels of endothelial MAO-B such as primates and humans are, therefore, much more sensitive to MPTP insult than species whose cerebral capillaries have large amounts of MAO-B such as rats and mice [22].

Among the 'classical' drug-metabolizing enzymes, cytochrome P450 haemoproteins, several cytochrome P450-dependent monooxygenases, NADPH-cytochrome P450 reductase and epoxide hydrolase activities have been detected in microvessel preparations from both human and rat brain tissue [17,20,23,24]. Cytochrome P450 IIE1 has been demonstrated by immunohistochemistry in most small blood vessels of the brain as well as in the glial end-feet abutting on the vessels. The immunoreactivity of blood vessel walls is not uniform among all brain areas, being especially prominent in areas such as the white matter of the cerebellum [7], which is not a highly vascular structure [25,26]. Cytochrome P450 IIB1,2 immunoreactivity is also strong in the cerebral microvessels. Both endothelial cells and pericytes are apparently stained [6].

As for conjugating enzymes, UDP-glucuronosyltransferase activity toward planar molecules such as 1-naphthol has been demonstrated in rat brain capillaries [20,23], and one α-class glutathione *S*-transferase (Y_kY_k) has been localized to endothelial cells and/or astrocytic end-feet associated with blood vessels [27]. More precise cellular localization of the enzymes seen in isolated brain capillary preparations has not been possible to date. Indeed because the basement membrane is continuous around the capillaries and encloses the capillary-associated pericytes, the isolated material includes not only the endothelial cells but also pericytes and astrocytic processes. Therefore caution has to be used in attributing 'isolated microvessel' activities to endothelial cells only. Be that as it may, the activities of some drug-metabolizing enzymes are several times higher in the microvessel than in the cortical parenchyma, and the level in microvessels can be increased by in vivo treatment with exogenous inducers [20,23]. These various observations highlight the potential for drug metabolism by the BBB and the influence this process may have on the entry of drug and toxins into brain.

A large part of the cytochrome P450 in cerebral microvessels is mitochondrial [23], and endogenous substrates have been identified for some cytochrome P450 isoenzymes [28]. As said before, it is likely that some isoenzymes of cytochrome P450 as well as some isoenzymes of other drug-metabolizing systems are involved in various physiological activities in the BBB under normal conditions.

The most striking finding concerning drug-metabolizing enzymes at blood-brain-CSF interfaces is the very high activities of UDP-glucuronosyltransferase and epoxide hydrolase in the rat choroid plexuses (Table 2), and to a lesser extent in the leptomeninges. Specifically, the activities of these enzymes in the choroid plexuses are at least equal to those in the liver when expressed on a per mg protein basis. The activities of several cytochrome P450 isoenzymes involved in the metabolism of xenobiotics are also relatively high in the choroid plexuses, although not as large as in the liver. The choroid plexuses and pial/arachnoid membranes are strongly cytochrome P450 IIB1,2 immunoreactive [6]. Also significantly present in the choroid plexuses are α class and μ class glutathione *S*-transferases [20,27]. Finally the choroid plexuses have high levels of glutathione peroxidase, an enzyme that could transform and inactivate various peroxides [29]. Although inducibility properties of the choroidal enzymes are incompletely studied to date, preliminary data indicate that the UDP-glucuronosyltransferase isoenzyme that catalyzes the conjugation of 1-naphthol is inducible by polycyclic aromatic hydrocarbon in a similar manner to that of the hepatic enzyme [30]. The precise cellular localization (i.e. epithelial versus endothelial) of most

Table 2
Some enzymes involved in the protection against reactive molecules and displaying a high specific activity in the rat choroid plexuses

Enzymes	Sustrates	Ratio of activity compared to cortex homogenate
UDP-Glucuronosyl-transferase (isoform conjugating planar substrate)	Mostly phenolic coumpounds	200–400
Epoxide hydrolase (membrane-bound)	Reactive (intermediate) epoxides and arene oxides	35
Glutathione peroxidase	Hydrogen and lipid peroxides	13

From [20,29].

of these enzymes has not be defined; preliminary immunohistochemistry findings with an antibody raised against the soluble form of the epoxide hydrolase has shown a strong signal specifically associated with the cytoplasm of the choroidal epithelial cells (J. Szmydynger-Chodobska, pers. commun.).

These different levels of activities among the systems associated with the choroid plexuses suggest that the drug-metabolizing enzymes in this structure may act not only as enzymatic barriers towards circulating exogenous molecules but also as 'liver-like' detoxification systems for the brain and CSF. This adds an additional pharmacotoxicological element to the various newly discovered roles of these peculiar cerebral structures (for further details, see the recent review by Johanson [15]). The site of metabolite secretion from the choroid plexuses (endothelium or epithelium; basolateral or apical membranes) and the mechanism of this processes remain to be studied.

4. Role of blood-brain interfaces in the elimination of intracerebrally formed metabolites

The metabolites of xenobiotics usually are more polar than the parent molecules. Because tight junctions seal most of the cells forming the blood-brain-CSF interfaces (i.e. capillary endothelial cells, choroidal epithelial cells, and arachnoid membrane cells), the elimination of such hydrophilic molecules from brain and CSF remains an issue, as just mentioned. In more detail, such metabolites may leave the brain by a specific transport mechanism at the level of the BBB, as has been shown for some glucuronides [19]. Alternatively, they may move from brain parenchyma into the ventricular or subarachnoid CSF by diffusion through the interstitium or bulk flow through the perivascular spaces. Recent evidence suggests that CSF and entrained material flow rapidly through the ventricles and aqueduct and into the lateral recesses of the fourth ventricle and subarachnoidal cisterns (Ghersi-Egea, Patlak, and Fenstermacher, unpublished observations).

As CSF flows along this route, exchange with the brain parenchyma can occur through the ependymal lining of the ventricles [31] and through the pial-glial border at some but not all places along the subarachnoid system (Ghersi-Egea and Fenstermacher, unpublished observations). With respect to the former site of CSF-brain exchange, drug-metabolizing enzymes such as the μ form of glutathione *S*-transferase [32], cytochrome P450 IIE1 [7] and cytochrome P450 IIB1,2 [6] have been reported to be present in relatively large amounts in the ependyma. With respect to the pial-glial membrane, the movement of polar substances from the subarachnoid space into brain parenchyma seems to be greatly reduced by a thick glia limitans (Fig. 1) at a

number of superficial midbrain structures such as the superior colliculus (Fenstermacher and Ghersi-Egea, unpublished observations) but is significant and apparently diffusional at other sites, for example, the cerebral cortex [33]. Ultimately, some or all of the CSF-contained metabolites will be cleared through the arachnoid villi into the venous system or into the cervical lymphatics via perivascular spaces [34].

5. Toxicity induced by drug metabolism in blood-brain interfaces

In some cases, highly reactive, accordingly toxic molecules, such as oxygen and xenobiotic radicals and electrophilic products, can be generated from the interaction of exogenous molecules with drug-metabolizing enzymes. For instance, cytochrome P450 IIE1 is responsible for the metabolic activation of several precarcinogens and solvents. Cytochrome P450 of the 1A family, together with epoxide hydrolase activates polycyclic aromatic hydrocarbons into highly reactive diol-epoxide metabolites. The consequences of such metabolic activation-induced cell toxicity could disrupt the integrity of the BBB and/or the choroidal barrier and ultimately lead to widespread brain dysfunction. Evidence that a production of superoxide anion occurs during the monoelectronic reductive metabolism of various drugs by brain capillaries [35] suggests that this 'negative' aspect of drug metabolism at brain interfaces is worthy of further investigation.

Among the currently available in vivo BBB models, a co-culture of bovine brain endothelial and glial cells that closely mimics the in vivo properties of cerebral capillaries has been developed by Dehouck et al. [36] and used to investigate the effects of hypoxia at the level of the BBB [37]. In this study, the co-cultured cells proved to be structurally and functionally altered by this model of oxidative stress, possibly by the production of reactive species. Among the alterations were an increase in permeability of the 'barrier,' rearrangement of the intracellular F-actin filaments associated with the 'tight' junctions of the endothelial cells, and a decrease in the activities of anti-oxidant enzymes. In vivo corroboration of this work and further in vivo and in vitro studies of the mechanisms and toxicological consequences of drug-induced metabolic activation at the level of the BBB are clearly called for in view of the importance of the BBB in various age-related neuropathologies such as dementia and multiple sclerosis.

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Localization and characterization of cytochrome P450 in the brain. In vivo and in vitro investigations on phenytoin- and phenobarbital-inducible isoforms

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Abstract

The antiepileptic drug phenytoin is known to be substrate as well as inducer of cytochrome P450 (P450) in the mammalian liver. We were able to show the expression of P450 species immunorelated to the main phenytoin-induced hepatic isoforms in mice (CYP2C29) and rats (CYP2B1,2) also in the central and peripheral nervous system and primary cultures of cell types from the brain. The 2B1,2 related protein showed only a weak constitutive expression in vivo and in vitro analyzed by immunocytochemistry, in situ hybridization, Northern blot and RT/polymerase chain reaction (PCR). Contrary, the CYP2C29 related form is inducible by phenytoin at about 1.5-fold starting from an already higher constitutive level. This protein is characterized by a remarkable tendency to dissociate from the endomembranes during tissue homogenization. The supernatant of microsomal pellet is able to metabolize phenytoin in a reconstitutive system.

Keywords: Blood-brain barrier; Brain metabolism; Cytochrome P450; Liver; Phenobarbital; Phenytoin

1. Phenytoin works as anticonvulsant, but provokes neurotoxic effects

Chronic application of antiepileptic drugs may cause cerebellar dysfunctions and in rare cases cerebellar degeneration. In the case of phenytoin it is not yet clearly understood whether the drug alone, the enormous metabolic stress during seizures or both are responsible for the cerebellar damage. Repeatedly we described significant dystrophic changes in presynaptic portions of Purkinje cell axons following long-term administration of phenytoin to mice [1]. In a present paper [unpublished] we have summarized all the aspects of morphological, ultrastructural, biochemical and electrophysiological alterations during phenytoin influence and subsequent withdrawal of the drug. We interpreted the presynaptic Purkinje cell axonal alterations as a consequence of a profound cerebellar functional network imbalance resulting in Purkinje cell hyperactivity with subsequent synaptic exhaustion and disintegration.

The pharmacological as well as the toxicological effects of phenytoin in this context are thought to be based on its accumulation in the membrane system of especially sensitive cells (neurons and glia cells) influencing their excita-

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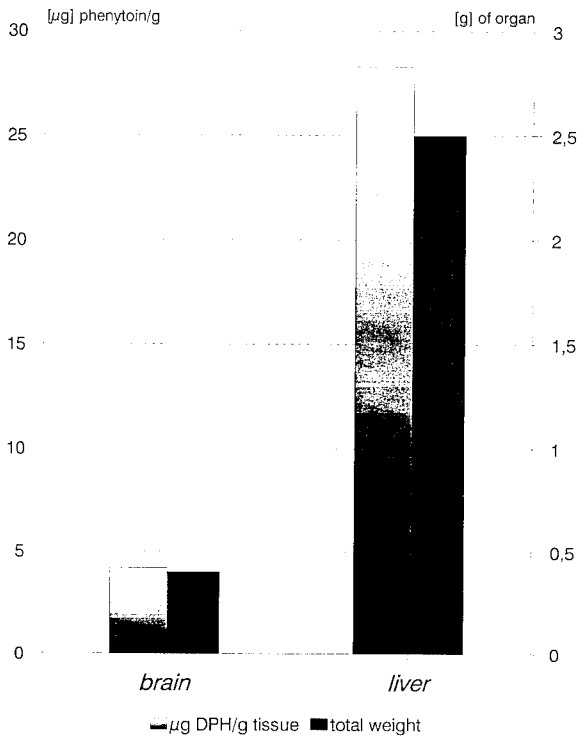


Fig. 1. Phenytoin (DPH, diphenylhydantoin) distribution in liver and brain tissues of mice following drug administration of a daily dose of 80 mg/kg body weight during 3 weeks. Comparison of phenytoin accumulation (μg DPH/g tissue) with the total body weight (g).

tion behavior and therefore their function. To prove this idea, we analyzed the distribution of orally given phenytoin within perfused liver and brain tissue (Fig. 1). It is striking that the ratios of the drug concentrations in brain and liver and those of total organ weights are comparable. The choice of the C57Bl/6J mouse as suitable animal model results from our findings that mice, contrary to rats, are less potent metabolizers of phenytoin. This results in a 4 times higher drug concentration in the serum (20 μg/ml) and neurotoxicological effects on cerebellar network structure and functions.

2. Phenytoin acts as substrate and inducer of distinct cytochrome P450 isoforms in the liver

Ahead of our investigations concerning the brain's capacity to metabolize phenytoin we had to clarify, above all, the role of the liver during the metabolization of the incorporated drug. Besides phenytoin we involved phenobarbital into our analyses. It has been evidenced in a scoparone/scopoletin/isoscopoletin assay [unpublished] that phenytoin behaves like phenobarbital with respect to cytochrome P450 (P450) induction. The competition between both inducers/substrates could therefore explain difficulties in the therapy of epileptic patients treated with a combination of phenytoin and phenobarbital [2,3]. In a rat model the enhanced degradation of phenytoin has already been shown when it was given in combination with phenobarbital [4].

Degradation of phenytoin or phenobarbital is initiated by P450 monooxygenase reactions in the liver [5]. Moreover, the drugs act species-dependent as inducers of P450 in rat and mouse liver (Fig. 2). The different amounts of total P450 are caused by differential expressions of specific isoforms induced by both drugs. Phenobarbital acts as potent inducer of CYP2B1 in rats [5] and the regioselective *O*-demethylation of scoparone is used as indicator for this kind of induction [6]. This metabolism, found also after phenytoin treatment, gives strong evidence for a phenobarbital-like induction of CYP2B1 by phenytoin in the rat liver. However, phenobarbital is the more potent inducer of CYP2B1 in rats.

In the mouse liver, phenytoin and phenobarbital act as inducers of P450 as well. But in contrast to the findings in rat liver, phenytoin is the more potent inducer (Fig. 3). The differences among the scoparone metabolites generated by liver microsomes of mice and rats, respectively, indicate the action of a novel P450 isoform in mouse, not comparable with the CYP2B1. This isoform has been purified and identified according to its *N*-terminal sequence as CYP2C29. Metabolic activity and drug clearance by the highly inducible CYP2B1 in rat liver seems to be more effective than those by CYP2C29 in mouse liver. This might be the reason for the extrahepatic effects of the xenobiotics found in mice, especially in the central nervous system (CNS). Hippocampal blebs [7], ataxia and Purkinje cell axonopathy [8] (see above) and first evidence for P450 action after phenytoin administration [9,10]

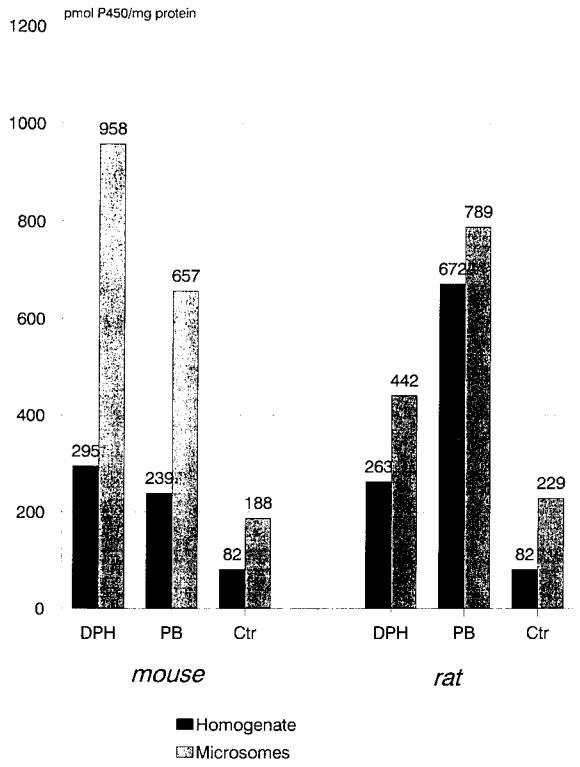


Fig. 2. Total content of P450 in subfractions of mouse and rat liver following administration of phenytoin and phenobarbital (see Fig. 1 for animal feeding). PB, phenobarbital; Ctr, untreated animals.

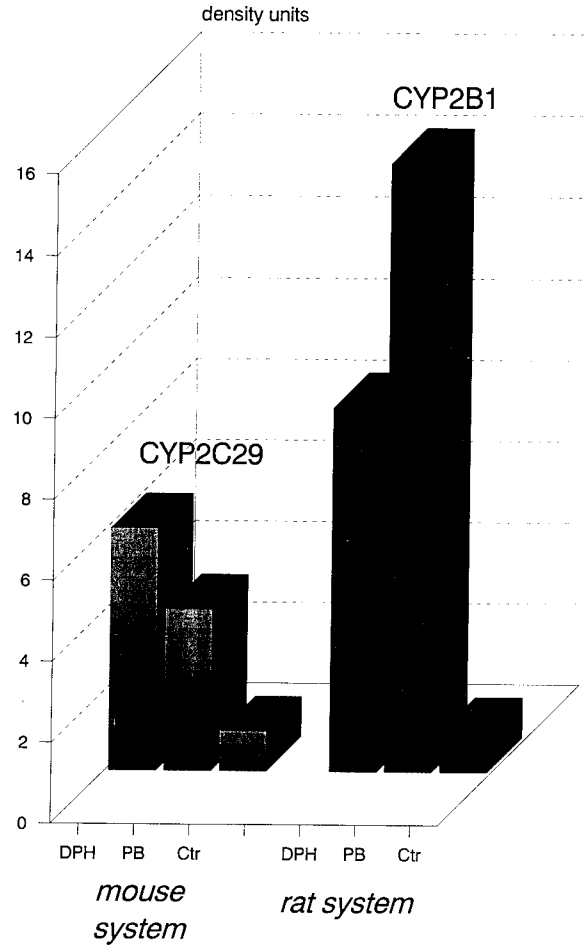


Fig. 3. Species differences in the P450 expression on the basis of different P450 isoforms induced in the liver tissues of rats and mice by DPH and PB. The detection of P450 isoforms was done with specific antibodies (see text) using Western blot and densitometric evaluation of the specific signals.

could be detected in the mouse brain, but not in the rat brain.

During the degradation of phenytoin by hepatic P450 enzymes short-living reactive metabolites are generated, which are likely candidates of the drug's cytotoxicity in general (Fig. 4). However, we are more interested in getting detailed information about the degradation of this antiepileptic drug in the brain.

3. Expression of P450 isoforms in the brain immunorelated to hepatic CYP2B1,2 and CYP2C29

3.1. Evaluation of total P450 content

First, the analysis of the total P450 content in brain tissue homogenates and microsomal fractions revealed a different increase following phenytoin and phenobarbital treatment, respectively (Fig. 5). Most striking is the high P450 value of the phenobarbital homogenate which has no equivalent on the microsomal level, whereas the phenytoin sample contains the higher P450 concentration. The supernatant of phenytoin and phenobarbital samples seems to be slightly enriched. The absence of the mitochondrial fractions may explain the fact that the P450 concentration in the homogenates is higher than in the microsomes.

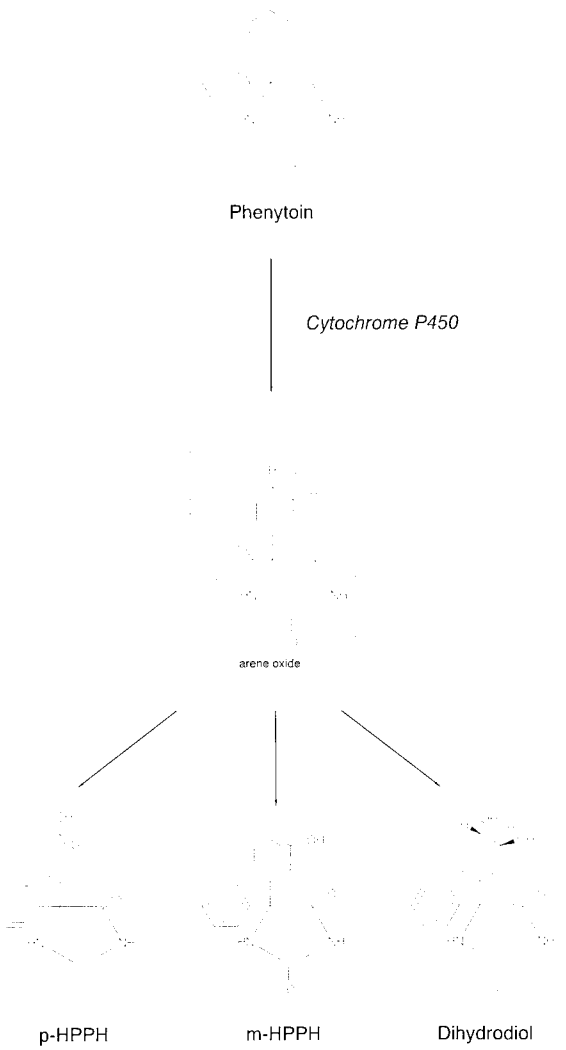


Fig. 4. The metablism of phenytoin by P450 results in the formation of 3 main degradation products via the short-living reactive metabolite arene oxide.

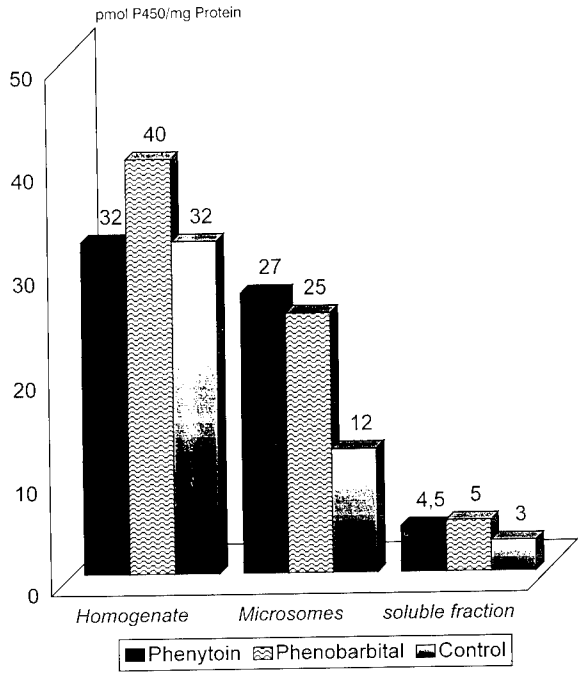


Fig. 5. Total content of P450 in subfractions of the mouse brain as measured by Na-dithionite-reduced carbon monoxide spectrophotometry.

3.2. Mapping of CYP2C29 and CYP2B1 immunorelated isoforms

Both isoforms, respectively, were used as immunogens to produce polyclonal antibodies as probes to investigate the nervous system, the target tissue of both antiepileptics. By means of these antibodies we could localize the sites of corresponding immunoreactivities throughout the nervous system [10]. The pattern of staining is not distributed evenly throughout the CNS. It was found to be restricted to only some cellular populations. The most striking aspect of immunostaining is a predominant reactivity in the evolutionary old brain parts. Neuropil and neuronal staining was found in the spinal cord (motor neurons of the ventral horn), medulla oblongata (hypoglossal nuclei, magnocellular part of the lateral reticular nuclei), pons (trigeminal, facial, cochlear and pontine nuclei), cerebellum (granule cells), midbrain (dorsal raphe nucleus) and limbic lobe (hippocampal pyramidal cells). Neuropil reactivity alone appeared in cerebellar nuclei, midbrain, thalamus, basal ganglia, neopallium and olfactory brain. Generally, pia mater/arachnoid, ependyma, choroid plexus, vascular system and some astrocyte populations were found to be strongly immunoreactive to antiCYP2C29 but less to antiCYP2B1,2. These structural elements are cellular components of interfaces: vascular and ventricular border lines. The cerebrovascular system has developed a unique mechanism to protect nerve cells against foreign substances

possibly acting as neurotoxins. This protection is made possible by the cooperation of structural and functional components of the blood-brain barrier. Part of the metabolic capacities of the cell types forming the body's border line are P450-directed hydroxylations of lipophilic substances accumulated in the endomembranes of endothelial cells, as well as accompanying pericytes, astrocytes and ependymal cells of the choroid plexus.

3.3. Analyses of P450 protein induction, purification and metabolic activities

To get more insight into cell type specific equipment with and properties of CYP2B1.2 and CYP2C29 related isoforms we turned to in vitro cultures of cell populations characterized by in situ P450 immunoreactivity. Primary cultures of endothelial and neuronal cells of astrocytes and microglia were found to be able to express both isoforms constitutively.

Brain capillary endothelial cells from pigs expressed CYP2B1,2 with striking cell-type differences up to day 10 in culture, as shown by immunocytochemical studies. Spectrophotometric analyses revealed a high content of total P450 in isolated endothelial cells, but the expression strongly decreased during 10 days of culture. The application of phenytoin to the medium (1 μM) caused no higher expression of P450, indicating that pig endothelial cells are not inducible under these conditions.

Contrary to these findings the results of phenytoin application to cultured astrocytes, the second cellular entity of the blood-brain barrier, had influence on the P450 content. Astrocyte cultures prepared from the rat brain showed a remarkable expression of both P450 species. The percentage of strongly immunoreactive astrocytes decreased from 40% after 7 days in culture to 15% after 21 days. Essentially all astrocytes have a low but significant P450 immunoreactivity within this interval. Phenytoin (1 μM) added to the medium for 7 days significantly (1.2-fold) evoked a significant increase of total P450 in astrocyte homogenates as confirmed by spectrophotometry and cell countings. Considerably more immunoreactive cells (1.5-fold) were found in treated cultures than in controls [11].

In the same way mouse astrocytes in culture express P450 with a low tendency to enhance its concentration following phenytoin and phenobarbital application to an extent comparable with that in brain tissue (see above, Fig. 5). Western blots of cultured astrocytes (controls and phenytoin-treated) showed a CYP2C29-immunoreactive protein in the molecular weight range of the corresponding hepatic immunogen (51–52 kDa) and additionally a stronger signal at 60–62 kDa (Fig. 6). This protein could be localized by preembedding immunoelectron microscopy as being associated with the endomembrane system, including smooth and rough endoplasmic reticulum, outer sheaths of the double-layered envelopes of mitochondria and nuclei and partly the limiting plasma membrane.

As in cultured astrocytes the weakly membrane-bound protein appeared very significant in the microsomal supernatant of rat and mouse brain tissue during differential centrifugation procedures. Attempts to purify this protein led to surprising insights in its uncommon solubility during tissue homogenization. Most immuno-

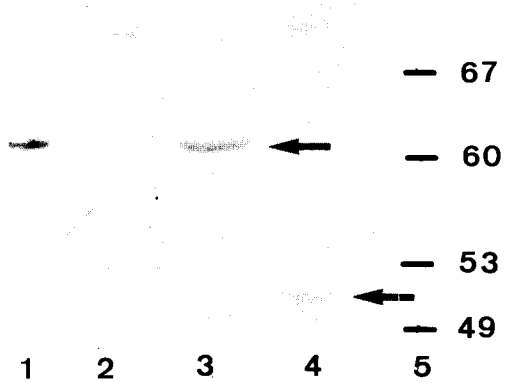


Fig. 6. CYP2C29 Western blot of phenytoin-treated (1,2) astrocyte primary culture compared with controls (3,4). (1,3) soluble fractions (150 000 × *g* supernatant); (2,4) membrane fraction (150 000 × *g* pellet); (5) marker proteins. The arrows point to the high-molecular-weight 'soluble' protein at 62 kDa and the typical CYP2C29 position at 51 kDa, respectively. The immunoreactivities against the antiCYP2C29 polyclonal antibody were labeled with the alkaline phosphatase reaction.

reactivity appeared in the supernatant of a 10 000 × g spin (containing membranes, mitochondria and cytosol). After the sedimentation of mitochondria (15 000 × g) and microsomes (100 000 × g) the signal accumulated further in the last supernatant. The faint signal of the microsomal pellet could be easily removed by sonication and a final microsome sedimentation. Such a sonication effect has not been observed in the case of the tightly membrane-bound CYP2B1,2 immunorelated isoform.

At present we are concerned with purifying and analyzing the 'soluble' protein. We established a reconstitutive test system which was able to metabolize ^{14}C-labeled phenytoin (Fig. 7). Further, the protein was enriched in a gradual isolation procedure by ammonia sulfate precipitation, hydrophobic interaction chromatography and preparative isoelectric focusing. It showed the characteristic dithionite-reduced carbon monoxide absorption peak at 450 nm and the Western blot signal at M_r 62 kDa corresponding to that in neuronal and glial subfractions. Further efforts have to be undertaken to reveal the identity of this protein. Molecular biological methods are a particularly suitable tool to realize this task.

3.4. Molecular biology

We are analyzing the mRNA expression in phenytoin-treated mouse astrocyte cultures by means of CYP2C29 probes as was done already with the CYP2B1 mRNA in rat astrocytes. In this case no signals could be detected on Northern blots. Contrary to this, RT/polymerase chain reaction (PCR) enabled the amplification and detection of a CYP2B1 specific sequence on Southern blots [unpublished]. The semi-quantitative estimation of the PCR products points to a low constitutive expression, but a lacking inducibility of the CYP2B1 gene in rat astrocytes by phenobarbital (Fig. 8).

The probes used for the detection of specific PCR products were applied to localize the CYP2B1 mRNA in single cells of the rat astrocyte cultures and in sections of rat brain and liver tissues. In situ hybridization with digoxigenin-labeled sense and antisense probes showed with convincing clearness the distribution of CYP2B1 mRNA as identical to the

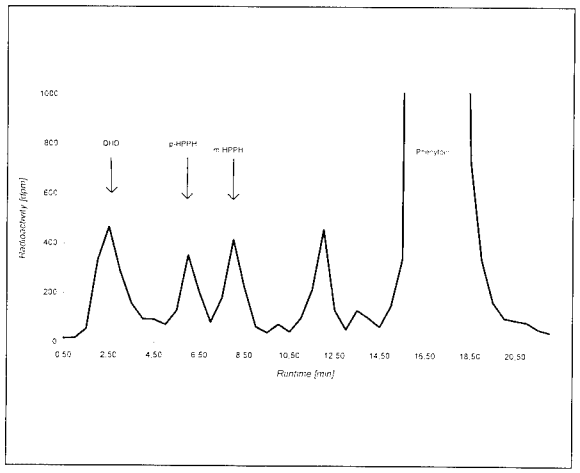


Fig. 7. The main phenytoin metabolites DHD (dihydrodiol), *p*-HPPH (*p*-hydroxyphenylphenylhydantoin) and *m*-HPPH (*m*-hydroxyphenylphenylhydantoin) generated by the soluble fraction of mouse astrocyte cultures (supernatant of the 150 000 × g spin). HPLC-radiogram of the reconstitutive assay supplemented with 0.25 μCi [^{14}C]phenytoin.

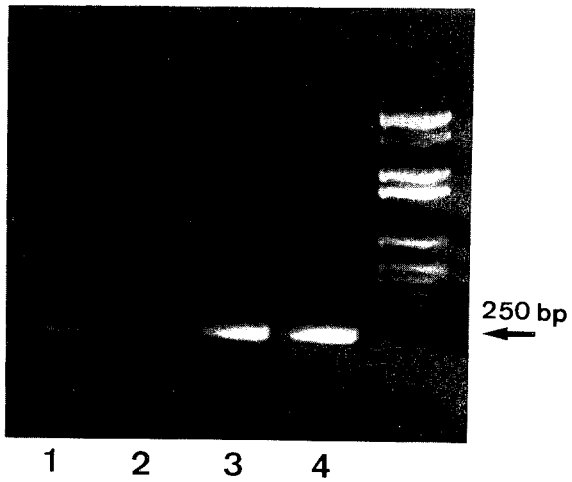


Fig. 8. CYP2B1-specific mRNA sequences could be detected in non-treated astrocyte cultures of rat brain and in the control liver tissue of rats following RT/PCR and Southern blotting. Ethidium bromide staining, 1.5% agarose gel electrophoresis. Lanes 1 and 2, astrocyte culture; lanes 3 and 4, liver tissue.

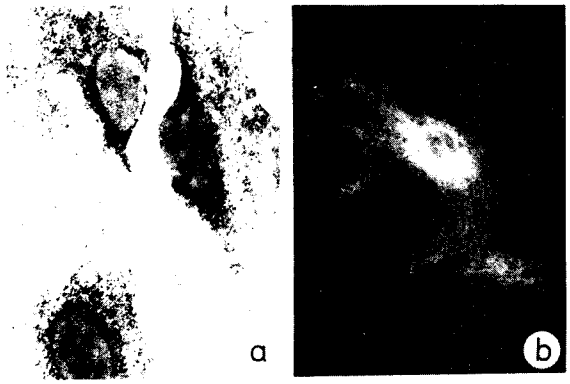


Fig. 9. (a,b) Astrocytes in culture were proved to express constitutively CYP2B1 mRNA (a) and protein (b). (a) In situ hybridization with digoxigenin-labeled antisense CYP2B1 cRNA-probes revealed the location of specific mRNAs in the perinuclear space. The detection of the hybridization events was performed by means of alkaline phosphatase immunocytochemistry. Magn.: ×630. (b) Immunocytochemical evidence of a CYP2B1,2 protein with the intracellular distribution comparable with those of CYP2B1 cRNA. Indirect fluorescence microscopy with fluorescein isothiocyanate. Magn.: ×400.

CYP2B1,2 protein distribution immunocytochemically labeled with the specific antibody. Even the intracellular labeling pattern is comparable because both macromolecules are concentrated on the perinuclear areas. The processes of the cells remain free of staining (Fig. 9).

Further attention will be focused on the full-length sequencing of brain P450 species and the analysis of task-sharing among the isoforms expressed in the various cell types of the CNS.

4. P450 monooxygenases form the key enzyme system in the chemical defense, but also in the regulation of endogenous substrates in the context of neuronal differention

The constitutive expression of P450 mRNA and protein independent of a possible induction by adequate substrates is a very important property of the cells of the nervous system. Therefore contrary to cultivated hepatocytes brain cells (neurons, endothelial and glia cells) retain their ability to express P450 in culture during 3–4 weeks.

It is also remarkable that hepatic endothelial cells lack CYP2B1,2 and CYP2C29 obviously due to high levels of various constitutive and inducible members of the P450 superfamily in the hepatocytes themselves. The constitutive presence of both isoforms in the vascular system of the CNS, forming the blood-brain barrier, might be a necessary protection which is active any time in view of the limited regenerative capability of the brain and its susceptibility to the effects of a broad spectrum of neurotoxically acting xenobiotics and drugs.

Besides the great importance of some P450s as tools in the chemical defense, growing evidence was found during the last decade for the involvement of the P450 system in the regulation of central nervous biochemical pathways. In particular the modification of gonadal and adrenal hormones in the brain (testosterone into estradiol) by CYP19 (aromatase), the synthesis of brain specific neurosteroids (cholesterol into pregnenolone) and the degradation of free steroids by specific P450 isoforms are central events and therefore decisive impulses for the early differentiation of many brain structures.

Both aspects of P450 activities in the nervous tissue are of relevance for our efforts to learn more about the structure and function of the brain.

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Hypomethylation of DNA: a nongenotoxic mechanism involved in tumor promotion[1]

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Abstract

There is an abundant amount of information on the mechanisms of action of genotoxic chemicals that act as carcinogens and the role that mutations play in carcinogenesis. However, carcinogenesis is more than mutagenesis and many carcinogens are not mutagens. Thus, there is a need to consider nongenotoxic mechanisms that may be involved in carcinogenesis. In this paper, we review our working hypothesis that hypomethylation of DNA is an epigenetic, nongenotoxic mechanism that plays a role in tumor promotion by facilitating aberrant gene expression. The utility of employing experimental models that focus on relevant comparisons between sensitive and resistant strains of mice is emphasized. Additionally, aspects of DNA methylation in rodents and humans are compared and contrasted. We discuss hypomethylation of DNA as a secondary mechanism, that is expected to be threshold-exhibiting, and conclude by describing how this information may facilitate a rational approach towards risk assessment when dealing with nongenotoxic compounds that are carcinogenic in a bioassay.

Keywords: Bioassay; Carcinogenesis; DNA methylation; Epigenetic; Genotoxic; Nongenotoxic; Promotion; Risk assessment

1. Introduction

Carcinogenesis is a multistep process that can be viewed as involving stages of initiation, promotion and progression [1]. The existence of these stages was, in our view, correctly not intended to connote that mutation only occurs during initiation, or that promotion only involves stimulating proliferation of and/or inhibiting apoptosis of initiated cells [2]. Operationally, promotion entails the clonal expansion of initiated cells and an initiated cell is one that may proliferate in response to promoter treatment. The terms initiation and promotion suggest different modes of action. Alterations to the genome, e.g. a change in DNA methylation (the extent and pattern of cytosine residues present as 5-methylcytosine (5MeC)) or mutation, take place at multiple points in the carcinogenic process, for example as in the model of colon carcinogenesis based on the work of Vogelstein et al. [3]. Carcinogens that interact with and alter DNA are categorized as genotoxic (the chemical or a metabolite of it is DNA reactive) and these are mutagenic in appropriate test systems [4].

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There is an abundant amount of information on mechanisms of action of genotoxic carcinogens and the role that mutations play in carcinogenesis [5]. However, carcinogenesis is more than mutagenesis and many carcinogens are not mutagens. A mismatch repair deficiency has been identified in phenotypically normal human cells. The people who donated these cells had numerous mutations in a variety of tissues but very few tumors were evident [6]. Additionally, among a group of 138 chemicals deemed to be carcinogenic as a result of bioassays conducted by the National Toxicology Program, 33% are not mutagenic to *Salmonella* and possess a non-alerting chemical structure [7]. Thus, there is a need to consider nongenotoxic mechanisms that play a role in carcinogenesis. Hypomethylation of DNA (a decrease in the level of 5MeC) appears to be one of these, and we are testing the hypothesis that hypomethylation of DNA is an epigenetic mechanism involved in tumor promotion [8].

2. Inheritance of phenotype, epigenetics and DNA methylation

Changes in gene expression resulting in an altered phenotype may be due to genetic mechanisms involving an altered DNA base sequence (i.e. mutation) or to epigenetic mechanisms involving heritable changes in DNA modification. The preservation of DNA methylation patterns and DNA-protein complexes for multiple cell generations are examples of heritable modifications to DNA [9]. DNA methylation is a mechanism underlying epigenetics, the term describing the transmission of alternative states of gene activity in somatic cells [10]. Additionally, methylation may play a role in genomic imprinting; the preferential expression of either the maternal or paternal allele of a gene [10–12].

DNA methylation regulates gene expression [12] by influencing chromatin structure [13] and the methyl group influences the binding of transcription factors, either directly or indirectly by facilitating the binding of proteins that bind to methylated DNA [14]. Inhibition of transcription and DNA methylation are related directly, and this is not an all-or-none phenomenon [15]. Hypomethylation is necessary but not sufficient for expression. Thus, a hypomethylated gene can be viewed as having a higher potential/probability of being expressed as compared to a hypermethylated gene [16]. In this context it should be noted that even in the case of the mannose-6-phosphate/insulin-like growth factor II receptor (Igf2r) gene where methylation in an intron seems to be an imprinting signal required for expression, hypomethylation of the 5′ flanking region is required for expression [17]. Hypomethylation may be a nongenotoxic mechanism facilitating the aberrant gene expression involved in carcinogenesis [8,16,18,19]. For example, excessive expression of normal Ha-*ras* can transform cells [20] and the transforming ability of a variety of oncogenes is dependent upon their increased expression [21]. This is not unexpected because with the exception of tumor suppressor genes, a mutated gene must be expressed in order to affect phenotype.

3. DNA methylation and carcinogenesis

Changes in DNA methylation are a consistent finding in cancer cells [22,23]. Arguably, overall hypomethylation of DNA is the most consistent alteration of the genome that is observed in carcinogenesis. For example, hypomethylation has been demonstrated to occur as an early event in the development of colon cancer [3]. Additional support for the role of hypomethylation of DNA in carcinogenesis is found by comparing the level of methylation at various stages of several types of human cancers; overall, the level of methylation decreases progressively as one goes from normal tissue to benign tumors to primary malignancies to secondary malignancies [22]. A recent study has also demonstrated that the degree of hypomethylation increases with the grade of cervical neoplasia [24]. Additionally, placement of rodents on methyl-deficient diets results in liver tumors and it is associated with hypomethylation of DNA [25,26].

There are other roles for DNA methylation in carcinogenesis and these involve tumor suppressor genes and mutation. Regional hypermethylation may play a role in carcinogenesis by inhib-

iting transcription of tumor suppressor genes [27] and by facilitating genetic instability leading to loss of tumor suppressor genes [28]. Spontaneous deamination of 5MeC results in the generation of thymine and may lead to a C-G to T-A transition [29]. Further, in the presence of low levels of *S*-adenosylmethionine, DNA methylase may be able to catalyze the deamination of cytosine to form uracil, again leading to C-G to T-A transitions [30]. Thus, the high rate of mutation at CpG dinucleotides may be due, in part, to methyltransferase-mediated deamination [23]. These additional possibilities are compatible with our hypothesis regarding the role of hypomethylation of DNA in tumor promotion [8] and are not mutually exclusive. Indeed, it is reasonable to predict that, alone or in combination, these aspects of altered DNA methylation may be involved in the multistep process of carcinogenesis.

4. Marked hypomethylation may inhibit tumorigenesis

The results of two recent publications indicate that hypomethylation of DNA brought about by a reduction in DNA methyltransferase levels can inhibit tumorigenesis. These involve antisense RNA to DNA methyltransferase mRNA [31] or heterozygosity of the methyltransferase gene in combination with administration of the methyltransferase inhibitor 5-azacytidine [32]. Both of these experimental conditions are expected to result in marked hypomethylation. Since DNA methylation plays a critical role in development [11] and differentiation [10], we believe it is reasonable to propose that hypomethylation at an intermediate level does play a critical role in carcinogenesis while excessive hypomethylation may not be compatible with cell life; dead cells do not form tumors.

5. Cell proliferation and DNA methylation

Cell proliferation plays a key role in carcinogenesis [33]. This involves expansion of clones of initiated cells during promotion and progression. Additionally, cell proliferation may contribute to carcinogenesis by facilitating mutagenesis [34], including the point mutations resulting from spontaneous deamination of 5MeC and methyltransferase-mediated deamination of cytosine, discussed above. However, it would be a mistake to focus upon mutagenesis solely. Hypomethylation may occur during periods of DNA replication, and/or as a result of decreased maintenance methylation [35], the latter may be either due to a decreased capacity/fidelity of the maintenance methylase, or a decreased level of the methyl donor, *S*-adenosylmethionine. Therefore, alterations in DNA methylation, including hypomethylation [19], may be one mechanism underlying a role for cell proliferation in carcinogenesis. Additionally, hypomethylation may occur by an enzymatic removal of 5MeC and replacement with cytosine that is not linked to cell proliferation [12].

6. Hypomethylation of DNA can be viewed as a secondary mechanism

A secondary mechanism involved in carcinogenesis is defined as an indirect mechanism that can be shown to exist under conditions of the bioassay (e.g. chronic administration of maximum tolerated doses of the test chemical) and can alter the tumor incidence independent of the test chemical per se [36]. The practical significance of the distinction between primary and secondary mechanisms is that secondary mechanisms are expected to involve a threshold. Based upon the criteria outlined for a secondary mechanism [37], we suggest that hypomethylation of DNA can be described as a secondary mechanism involved in carcinogenesis. This view is supported because hypomethylation of DNA is a biologically plausible, nongenotoxic, threshold-exhibiting, mechanism that is involved in the regulation of gene expression. In addition, the level of 5MeC can be measured experimentally in specific genes and in a global sense in entire tissues. Hypomethylation of DNA has been demonstrated in human and animal cancers. A particularly exciting aspect of our hypothesis is the data indicating that conditions which favor methylation can inhibit carcinogenesis, e.g. treat-

ment with *S*-adenosylmethionine decreased the incidence, number and size of preneoplastic lesions and hepatocellular carcinomas in rats that had been subjected to initiation with diethylnitrosamine and promotion with phenobarbital [38]. Taken together each of these points provides support for considering hypomethylation of DNA as a secondary mechanism involved in tumorigenesis.

7. Mouse liver tumorigenesis as a model

Our experimental interests involve testing the hypothesis that hypomethylation of DNA is a nongenotoxic mechanism that facilitates aberrant gene expression involved in tumor promotion [8]. 5MeC most commonly occurs at CpG sites, but may also occur at CpC, CpA, or CpT sites. Additionally, 5MeC may be found in mammalian cells at CXG sites [39]. Methylation status of DNA is generated by a maintenance process that occurs after DNA replication using the 5MeC present on the parental strand as a signal [12]. However, methylation of DNA is not limited to the maintenance methylation reaction, and may also occur de novo [12]. This process provides a mechanism whereby hypomethylation may be reversed and reversibility is a hallmark feature of the promotion stage of carcinogenesis [1]. Specifically, our research [8] is focused on an examination of methylation status and gene expression in strains of mice that have different susceptibilities to both spontaneous and chemical-induced liver tumorigenesis; the B6C3F1 and C3H/He are relatively susceptible strains, while the C57BL/6 is relatively resistant [40]. Importantly, the B6C3F1 is the F1 hybrid of a C57BL/6 female and a C3H/He male, so comparisons may be made between genetically related animals.

Our initial focus on Ha-*ras* was due to the high, though not universal, involvement of mutated Ha-*ras* (at codon 61) in genotoxic chemical-induced and spontaneous liver tumors in the B6C3F1 mouse [41,42]. The C3H/He, also a relatively sensitive strain, has a variable incidence of mutation of Ha-*ras* in spontaneous liver tumors [42,43]. Nongenotoxic chemical induced liver tumors in the B6C3F1 mouse [44] and the C3H/He mouse [45] have a low frequency of mutated Ha-*ras*. However, as discussed below, this does not preclude hypomethylation and increased expression of Ha-*ras* playing a role in the development of these tumors. Interestingly, non-liver tumors in the B6C3F1 mouse have a low incidence of Ha-*ras* mutation; as do diethylnitrosamine-induced liver tumors in the Fischer-344 rat [41] and the C57BL/6 mouse [42]. Thus, the lack of uniformity in detecting mutation of Ha-*ras* in these tumors suggests that a factor(s) in addition to mutation may influence tumor formation. It was in this context that we assessed methylation status and gene expression of Ha-*ras* in normal mouse liver and in liver tumors. Our studies demonstrated that Ha-*ras* in the liver tumor prone strains (i.e. B6C3F1 and C3H/He) lacks a methylated site that is present in the liver tumor resistant C57BL/6 [16]. This gene was also shown to be hypomethylated in chemical-induced (using genotoxic and nongenotoxic compounds) and spontaneous liver tumors in the B6C3F1 mouse [16,46]. Increased expression of Ha-*ras* was also evident [16,47]. Together these data demonstrate that hypomethylation and increased expression of Ha-*ras* are common events in liver tumorigenesis in the B6C3F1 mouse. Furthermore, we have suggested [47,48] that those hepatocytes exhibiting an increased expression of Ha-*ras* and *raf* may be able to overcome the growth inhibitory actions of phenobarbital (PB) and serve as the progenitors of PB-promoted liver tumors. Indeed, development of resistance to growth inhibition may be a general mechanism involved in tumor promotion as opposed to being unique to PB [48].

In light of the multistep nature of tumorigenesis we did not limit our analysis to one gene involved in control of cell growth and differentiation; we also investigated the *raf* oncogene as another potential target. Ha-*ras* and *raf* are both involved in signal transduction with *raf* functioning downstream of, or independently of, Ha-*ras* [49]. We have demonstrated hypomethylation of *raf* in the B6C3F1, but not the C57BL/6, after a promoting dose of PB for 2 weeks [47]. Additionally, *raf* is hypomethylated in the B6C3F1 mouse in spontaneous and PB-induced liver

tumors [47]. Increased *raf* expression is evident in the majority of PB-induced, but not spontaneous, liver tumors [47]. Taken together, these data support the notion that PB-induced tumors develop along a different pathway from spontaneous tumors and that the C57BL/6 has a higher capacity to maintain normal methylation status of DNA than the liver tumor prone B6C3F1.

Interestingly, a murine transgene has been demonstrated to be differentially methylated depending upon the genetic background of the host strain. There is a high level of methylation and lack of expression when the C57BL/6 mouse serves as the host, while the gene is unmethylated and expressed in the DBA/2 mouse [50]. DBA/2 mice are approximately 20-fold more susceptible to hepatocarcinogenesis than C57BL/6 mice following perinatal treatment with diethylnitrosamine [51]. This supports our view [8,47,48,52] that hypomethylation is involved in carcinogenesis and that a low capacity to methylate DNA can contribute to an increased susceptibility to carcinogenesis.

8. 5′ Flanking region of Ha-*ras*: mouse vs. human

We have analyzed the methylation status of portions of the 5′ flanking region of Ha-*ras* in B6C3F1 and C57BL/6 mice by two approaches, (1) Southern analysis utilizing oligonucleotide probes [53] and (2) a more sensitive approach utilizing pre-PCR digestion of DNA with methylation sensitive enzymes [54]. Our data demonstrate the presence of 5MeC in a portion of this region that contains a low frequency of CpG (region JC5/8 in Fig. 1), while the region with a high CpG frequency (observed/expected >0) is unmethylated (region JC6/7 in Fig. 1). Importantly, the JC6/7 region exhibits the main characteristic of a CpG island, that being a CpG ratio that is greater than the expected frequency in DNA, and because of this is expected to be unmethylated [55]. The JC5/8 portion, on the other hand, does not share this characteristic and is not expected to be completely unmethylated. Additionally, we have shown that the B6C3F1 contains an unmethylated CCGG site that is

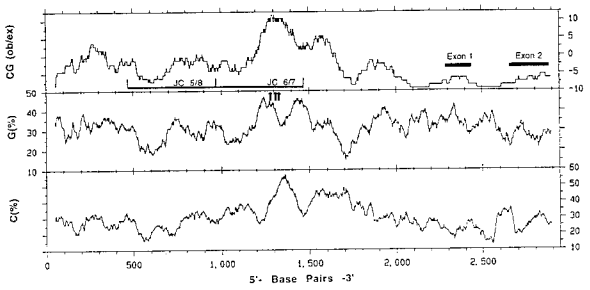


Fig. 1. Distribution of cytosine (C) and guanine (G) bases, and CpG dinucleotides in the 5′ flanking region of Ha-*ras*. The percent of C and G and the ratio of observed CpG to expected (ob/ex) is used to identify the presence of CpG islands (ob/ex >0), shown here for the 5′ flanking region of Ha-*ras*. The distribution was generated with the GCG package [56] of computer programs using 'Window' to carry out the analysis and 'Statplot' to generate the figure. The regions amplified by PCR primer sets JC5/8 (JC5: 5′-GCGACCGGGGTGAGCGTGCAA -3′; JC8: 5′-AGAGCCTCCACCCTGCAGCCT -3′) and JC6/7 (JC6: 5′-TGCCCGCACTCACCGCTTGCT -3′; JC7: 5′-AGGCTGCAGGGTGGAGGCTCT -3′) are noted, along with the positions of exons I and II. The arrows indicated the locations of the putative transcription start sites. The sequence information, 2700 base pairs, position of putative transcription start sites and exon locations were obtained from refs. [57,58].

methylated in the C57BL/6 and would suggest that this site may play a role in the high spontaneous liver tumor incidence in the B6C3F1 mouse [54].

Our interest in this nongenotoxic mechanism of tumor promotion prompted us to compare the sequences of the 5′ flanking region of Ha-*ras* between mice and humans using the GCG package [56]. The sequence comparisons are limited by the availability of the data in the literature. Comparison of the 5′ region of Ha-*ras* (to -330 bp from the transcription start site) between Sencar [57] and NMRI [58] mice reveals 99% sequence identity (Fig. 2). However, a comparison of this region between NMRI mice [58] and human Ha-*ras* [56] shows only ~59% sequence identity (Fig. 3). Furthermore, a comparison of this region (to -550 bp from the transcription start site) between Sencar mice [57] and human Ha-*ras* [59] reveals ~50% sequence identity (Fig. 4). This lack of sequence identity in the promoter regions of mouse and human Ha-*ras* suggests

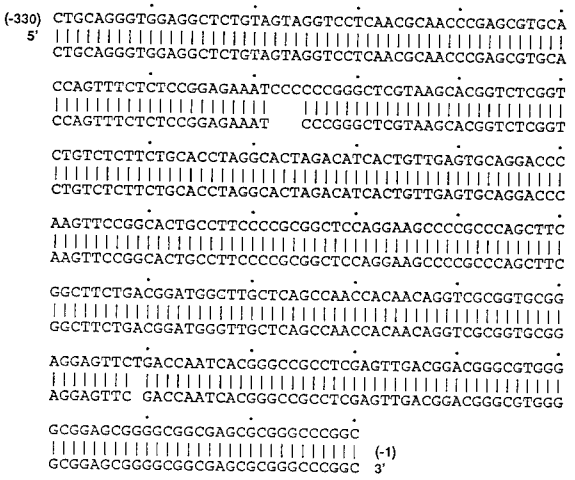


Fig. 2. A comparison of the base sequence of the 5′ flanking region of Ha-*ras* in SENCAR mouse and NMRI mouse DNA. The base sequence of Ha-*ras* in SENCAR mouse DNA (top strand) and NMRI mouse DNA (bottom strand) was compared using the 'Gap' program in the GCG package [56]. The numbering represents the distance in base pairs from the transcription start site located most 5′. Sequence data were compared for the SENCAR mouse Ha-*ras* [57] and NMRI mouse Ha-*ras* [58]. Our comparison was limited by the extent to which Brown et al. sequenced Ha-*ras* in the 5′ direction. A 99% similarity in the Ha-*ras* sequence was observed between the two strains of mice.

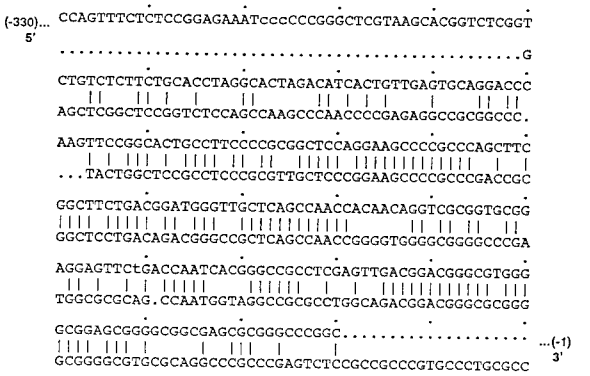


Fig. 3. A comparison of the base sequence of the 5′ flanking region of Ha-*ras* in NMRI mouse and human DNA. The base sequence of Ha-*ras* in NMRI mouse DNA (top strand) and human DNA (bottom strand) was compared using the 'Gap' program in the GCG package [56]. The numbering represents the distance in base pairs from the transcription start site located most 5′. Sequence data for the NMRI mouse Ha-*ras* [58] and human Ha-*ras* [59] were compared. Our comparison was limited by the extent to which Brown et al. sequenced Ha-*ras* in the 5′ direction. A 59% similarity in the Ha-*ras* sequence was observed between the mouse and human.

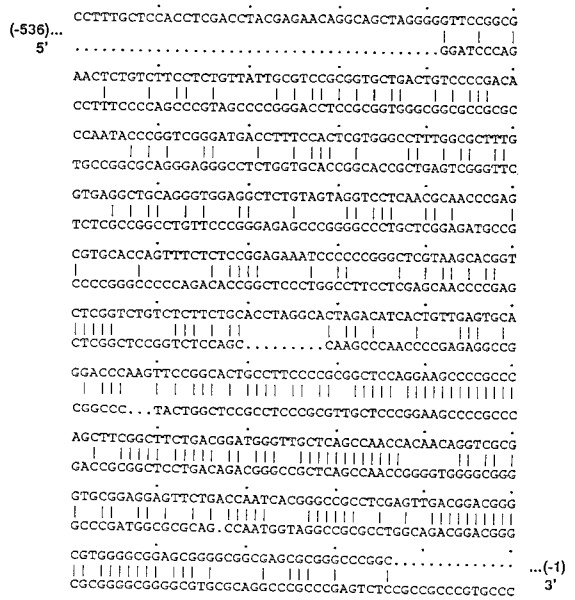


Fig. 4. A comparison of the base sequence of the 5′ flanking region of Ha-*ras* in SENCAR mouse and human DNA. The base sequence of Ha-*ras* in SENCAR mouse DNA (top strand) and human DNA (bottom strand) was compared using the 'Gap' program in the GCG package [56]. The numbering represents the distance in base pairs from the transcription start site located most 5′. Sequence data for the SENCAR mouse Ha-*ras* [57] and human Ha-*ras* [59] were compared. Our comparison was limited by the extent to which Ishii et al. sequenced Ha-*ras* in the 5′ direction. A 50% similarity in the Ha-*ras* sequence was observed between the mouse and human.

that the regulation of Ha-*ras* would not be the same in these two species as many important *cis* elements for transcription factor binding are located in the regions that we compared [53,60].

9. DNA methylation: implications for risk assessment

The testing of our working hypothesis, that hypomethylation of DNA is an epigenetic, nongenotoxic, threshold-exhibiting mechanism that facilitates the aberrant gene expression involved in tumorigenesis [8,19,48,52] is yielding a greater understanding of mechanisms of tumorigenesis. In addition, it provides the type of mechanistic information that is required to take a more rational approach to risk assessment

[61,62]. A recent evaluation of the use of data obtained from animal carcinogenicity studies for human risk assessment demonstrated the difficulty encountered when dealing with nongenotoxic compounds because these appear to produce cancer in a species- and/or dose-specific manner [63]. This reinforces our contention that a greater understanding of mechanisms/modes of action of nongenotoxic agents will provide the type of information required to make more meaningful comparisons between species.

Knowledge about a potential mechanism underlying the heightened sensitivity of the B6C3F1 mouse to liver tumorigenesis, e.g. a decreased capacity for and/or fidelity of maintenance methylation compared to the relatively resistant C57BL/6, provides insight about species-to-species extrapolation. That is, bioassay data from a strain of mouse uniquely deficient in the ability to maintain normal methylation status (e.g. the B6C3F1) may not be relevant for human risk assessment, while studies of methylation status in animals better capable of maintaining normal methylation status (e.g. the C57BL/6) may be more relevant to humans. In support of this notion, we are able to demonstrate global liver DNA hypomethylation in the B6C3F1 mouse, but not the C57BL/6, after 1 week of administration of a choline-devoid, methionine deficient diet. After 12 weeks of feeding a choline-devoid, methionine-deficient diet, sufficient methyl groups were removed to demonstrate a statistically significant level of global hypomethylation of liver DNA in both strains [64]. This suggests an important quantitative, rather than a qualitative, difference between these strains of mice.

In this context, it is illustrative to compare rodents and humans when one considers the relationship between capacity to maintain the nascent methylation status of DNA and susceptibility to tumorigenesis. In contrast to rodent cells, normal human cells in culture rarely undergo spontaneous transformation and have generally proven resistant to transformation by carcinogens [65]. This correlates with the observation that the methylation status of DNA in primary diploid cells in culture is maintained more faithfully in human than in rodent cells [66]. We are suggesting that hypomethylation is relevant to tumorigenesis in both rodents and humans, and that humans may be less susceptible than rodents due, in part, to a better ability to maintain nascent patterns of DNA methylation. In this regard, the liver tumor resistant C57BL/6 mouse may be more similar to humans than the sensitive B6C3F1 mouse.

We view hypomethylation of DNA as a secondary mechanism [37] that may provide insight leading to a more rational interpretation of animal studies for human risk assessment. Our research is providing practical information concerning dose-response relationships, including the existence of plausible thresholds, that can be used as a mechanistic and theoretical framework when considering a safety factor, or multiple of exposure, approach to risk assessment. In this regard, we envision an experimental approach towards analysis of methylation status as a component of the carcinogen bioassay where the no adverse effect level of a nongenotoxic compound may be considered the highest dose at which there is no hypomethylation in the potential target organ [8,67].

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Peroxisome proliferation: current mechanisms relating to non-genotoxic carcinogenesis

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Abstract

A wide variety of chemicals have been shown to produce liver enlargement, peroxisome proliferation and induction of peroxisomal and microsomal fatty acid-oxidising enzyme activities in rats and mice. Some peroxisome proliferators have also been shown to increase the incidence of liver tumours in these species. Rodent peroxisome proliferators are not considered to be genotoxic agents. Proposed mechanisms of liver tumour formation include induction of sustained oxidative stress, enhanced cell replication, promotion of spontaneous preneoplastic lesions and inhibition of apoptosis. Marked species differences in the effects of peroxisome proliferators have been observed in both in vitro and in vivo studies. Key issues concerning the risk assessment to humans of exposure to rodent peroxisome proliferators are discussed.

Keywords: Apoptosis; Cell replication; Hepatic peroxisome proliferation; Hepatocarcinogenesis; Oxidative stress; Peroxisomes; Promotion; Species differences

1. Introduction

Peroxisomes (or 'microbodies') are single membrane-limited cytoplasmic organelles which are characterised by their content of catalase and a number of hydrogen peroxide-generating oxidase enzymes [1–4]. Like mitochondria peroxisomes contain a complete fatty acid β-oxidation cycle [2,4]. This paper will focus on mechanisms of peroxisome proliferator-induced hepatocarcinogenicity in rodents and species differences in response.

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2. Peroxisome proliferation in rodent liver

The administration of peroxisome proliferators to rats and mice results in marked liver enlargement which is due to both hepatocyte hypertrophy and hyperplasia [1–3,5–9]. Table 1 summarises the major morphological and biochemical changes observed in rodent liver. Organelle proliferation is associated with a differential induction of peroxisomal enzyme activities. While the enzymes of the β-oxidation cycle (normally assessed as cyanide-insensitive palmitoyl-CoA oxidation) are markedly induced, only small changes are observed in other peroxisomal enzyme activities such as catalase and D-amino acid oxidase. Apart from stimulating peroxisomal fatty acid metabolism, peroxisome

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Table 1
Some characteristics of peroxisome proliferation in rat and mouse liver

Liver weight	
(1)	Liver enlargement due to both hepatocyte hyperplasia and hypertrophy
(2)	Increased replicative DNA synthesis (may be either transient or sustained)[a]
Morphological changes	
(1)	Increased number and size of peroxisomes
(2)	Many 'coreless' peroxisomes observed[b]
(3)	Increased smooth endoplasmic reticulum
(4)	Increased numbers of mitochondria
(5)	Lysosomal changes and lipofuscin deposition[a]
(6)	Liver nodules and hepatocellular carcinoma[a]
Biochemical changes	
(1)	Selective induction of peroxisomal enzymes (e.g. marked induction of peroxisomal fatty acid β-oxidation enzymes but only a small increase in catalase activity)
(2)	Induction of microsomal fatty acid (ω-1)- and particularly ω-oxidising enzyme activities (due to induction of cytochrome P450 isoenzymes in the CYP4A subfamily)
(3)	Induction of carnitine acetyltransferase activity
(4)	Increase in an 80 kDa molecular weight polypeptide (due to induction of component enzymes of the peroxisomal fatty acid β-oxidation cycle)
(5)	Induction of cytosolic epoxide hydrolase
(6)	Induction of microsomal bilirubin UDPglucuronosyltransferase
(7)	Inhibition of glutathione peroxidase, glutathione *S*-transferase and superoxide dismutase activities

[a] Depends on test compound, dose and duration of treatment.
[b] Normal rat and mouse liver peroxisomes contain a crystalline nucleoid core consisting of insolubilised urate oxidase.
For further details see Refs. [1–10].

proliferators also increase microsomal fatty acid (ω-1)- and particularly ω-hydroxylase activities. This is due to induction of cytochrome P450 isoenzymes in the CYP4A subfamily and is normally measured as lauric acid 12-hydroxylase [10]. Peroxisome proliferators also markedly increase carnitine acetyltransferase activity which is localised in peroxisomal, mitochondrial and microsomal fractions [6]. In rat liver good correlations have been reported between the induction of peroxisomal fatty acid β-oxidation and organelle proliferation and between the induction of peroxisomal and microsomal fatty acid-oxidising enzyme activities [6,9–11].

Several laboratories have demonstrated that the characteristics of peroxisome proliferation in vivo may also be observed in vitro in primary rat and mouse hepatocyte cultures. Indeed, hepatocyte cultures have been employed for studying various aspects of peroxisome proliferation including structure-activity relationships and species differences in response [5–9,11,12].

Recent studies have demonstrated that the induction of peroxisome proliferation in rodent hepatocytes is mediated through peroxisome proliferator-activated receptors (PPARs) [4,9,13]. The first PPAR (termed mPPARα) was isolated from mouse liver by Issemann and Green [13] and several PPARs have since been identified in the mouse, rat, *Xenopus* and human. PPARs are members of the steroid hormone receptor superfamily, contain putative ligand binding and DNA binding domains, and bind to DNA as a heterodimer with the retinoid X receptor (RXR). Peroxisome proliferator response elements have been found in genes for both peroxisomal and microsomal fatty acid oxidising enzymes [4,9]. In in vitro expression systems PPARs can be activated by both peroxisome proliferators and certain fatty acids (e.g. arachidonic, linoleic and oleic acids). Some PPARs may be dominant repressors of other forms [9,14]. Multiple PPARs, together with activating (e.g. RXR) and repressing receptors, may account for known tissue and species differences in response to peroxisome proliferators [4,9,13]. The importance of mPPARα in the induction of peroxisome proliferation in mouse liver has been recently demonstrated [15]. In these studies the ligand binding domain of mPPARα was disrupted by homolo-

gous recombination and the homozygous (−/−) mutant mice were shown to lack mPPARα mRNA and protein. While liver enlargement, peroxisome proliferation and induction of mRNAs for peroxisomal and microsomal fatty acid oxidising enzymes were observed in wild type (+/+) mice treated with peroxisome proliferators, such effects were essentially absent in the mutant mice [15].

3. Rodent peroxisome proliferators

Many different classes of chemicals have been shown to produce peroxisome proliferation in rat and mouse hepatocytes (Table 2). While peroxisome proliferators appear to be structurally diverse, at least for some compounds, similarities in their 3-dimensional structures and structure-activity relationships have been reported [9,11]. A characteristic feature of many, but not all, peroxisome proliferators is the presence of an acidic function [5,11]. This acidic function is normally a carboxyl group, either present as a free carboxyl group in the parent structure or one that is unmasked by metabolism. Alternatively, the chemical may contain a chemical grouping which is a bioisostere of a carboxyl group, such as tetrazole or a sulphonamide moiety [5,11].

It should be noted that rodent liver peroxisome proliferators exhibit marked compound potency differences. While potent peroxisome proliferators include compounds developed as hypolipidaemic agents (e.g. ciprofibrate, Wy-14,643), plasticizers such as di-(2-ethylhexyl) phthalate (DEHP) are less potent and chemicals such as acetylsalicylic acid are even less potent [9,11].

4. Carcinogenicity of peroxisome proliferators

Hepatic peroxisome proliferation is of importance, not only because of the large range of industrial and other chemicals which produce this effect in rodents, but because certain of these agents have been found to increase the incidence of liver tumours in rats and/or mice [1–3,6–9]. In addition, peroxisome proliferators may also produce tumours in other organs such as the pancreas and testis [16].

Although peroxisome proliferators can produce hepatocellular carcinoma in rodents, they are not considered to be genotoxic agents. Studies with several peroxisome proliferators have shown that they do not bind covalently to DNA after in vivo administration to rats and mice [6–9]. Peroxisome proliferators have been extensively tested in a wide range of short-term tests for mutagenic and genotoxic potential [8,17]. Generally peroxisome proliferators produce negative results in such tests, although some positive findings have been reported mostly in vitro [8,9,17]. In keeping with the properties of non-genotoxic rodent hepatocarcinogens, peroxisome proliferators do not produce tumours when examined in initiation studies [18]. However, when appropriate histochemical markers are employed, several peroxisome proliferators have

Table 2
Examples of classes of chemicals which produce peroxisome proliferation in rodent liver

Chemical class	Examples[a]
Therapeutic agents	Acetylsalicylic acid, bezafibrate, bifonazole, ciprofibrate, clobuzarit, clofibrate, DL-040, fenofibrate, methylclofenapate, LY 171883, nafenopin, tiadenol, Wy-14,643
Steroids	Dehydroepiandrosterone
Herbicides	2,4-Dichlorophenoxyacetic acid, fomesafen, lactofen, 2,4,5-trichlorophenoxyacetic acid
Plasticizers	DEHA, DEHP, di-(2-ethylhexyl)terephthalate, di-(isodecyl)phthalate, di-(isononyl)phthalate, tri-(2-ethylhexyl)trimellitate
Solvents and industrial chemicals	Chlorinated paraffins, perchloroethylene, perfluoro-*n*-octanoic acid, trichloroethylene
Food flavours and natural products	Cinnamyl anthranilate, citral linalool

[a] The examples include compounds that were either developed and not marketed or have been withdrawn.
For further details see Refs. [1–3,5,6–9,11].

been demonstrated to be effective in rat liver tumour promotion studies [18].

5. Mechanisms of hepatocarcinogenesis

Several hypotheses have been proposed to account for why peroxisome proliferators can produce liver tumours in rats and mice. These mechanisms include:

(1) induction of sustained oxidative stress to hepatocytes [2,19];
(2) a role for increased cell proliferation [6,18,20];
(3) the promotion of spontaneously formed preneoplastic liver lesions [18,21,22];
(4) inhibition of apoptosis [23,24];
(5) other mechanisms and/or a combination of any of the above [9].

The oxidative stress hypothesis is based on the observation that the chronic administration of peroxisome proliferators produces a sustained oxidative stress in rodent hepatocytes due to an imbalance in the production and degradation of hydrogen peroxide [2,19]. Peroxisome proliferators markedly induce the enzymes of the peroxisomal fatty acid β-oxidation cycle, but produce only a small increase in catalase activity. The first enzyme of the β-oxidation cycle, acyl-CoA oxidase, produces hydrogen peroxide and hence the cyclic oxidation of a single fatty acid molecule can result in the production of several molecules of hydrogen peroxide [6]. Any excess hydrogen peroxide not destroyed by peroxisomal catalase can diffuse through the peroxisomal membrane into the cytosol where it will be a substrate for cytosolic selenium-dependent glutathione peroxidase. However, this enzyme activity and that of other enzymes including superoxide dismutase and glutathione *S*-transferases are often reduced by the administration of peroxisome proliferators to rodents [6,7,9,19]. These enzyme changes are postulated to result in increased intracellular levels of hydrogen peroxide which, either directly or via reactive oxygen species (e.g. hydroxyl radical), can attack membranes and DNA [2,19].

A number of experimental observations have provided support for the involvement of oxidative stress in the hepatotoxicity of peroxisome proliferators [2,6,9,19]. For example, in some studies peroxisome proliferators have been shown to increase hepatic lipid peroxidation, produce lipofuscin deposition, to modulate levels of hepatic antioxidants and to increase levels of 8-hydroxydeoxyguanosine in hepatic DNA [6,7,9,19]. However, although evidence of oxidative damage to hepatocytes has been observed in some studies, the magnitude of such effects does not correlate with the potency of the compound to produce tumours [6,9]. Thus, the available data would suggest that sustained oxidative stress is unlikely to be solely responsible for peroxisome proliferator-induced hepatocarcinogenesis in rodents.

Many investigations have demonstrated that cell proliferation is an important factor in the development of tumours by both genotoxic and non-genotoxic agents [6]. For example, an enhanced rate of cell replication can increase the frequency of spontaneous lesions and the probability of converting DNA adducts from both endogenous and exogenous sources into mutations before they can be repaired. Peroxisome proliferators are known to produce a burst of cell replication in rodent hepatocytes during the first few days of administration [2,7,9] and in some studies peroxisome proliferators have also been shown to produce a sustained stimulation of replicative DNA synthesis [6,9].

Some investigations have demonstrated the presence of numerous foci of putative preneoplastic cells in the livers of untreated old rats and mice [21,22]. These lesions are considered to represent spontaneously initiated cells as they have similar biological characteristics to those of cells initiated by genotoxic carcinogens [22]. The ability of peroxisome proliferators to produce tumours in young compared to old rats has been investigated. Both nafenopin and Wy-14,643 have been reported to produce more adenomas and carcinomas in old as against in young rats [6,9].

Several studies have demonstrated that non-genotoxic carcinogens can affect apoptosis in

rodent liver. Nafenopin has been reported to inhibit the apoptosis that occurs in rat liver after withdrawal of a mitogenic agent [23] and to inhibit apoptosis in hepatocytes and a rat hepatoma cell line in vitro [24]. The suppression of apoptosis by peroxisome proliferators may be important in liver tumour formation in that the growth of transformed cells may not be prevented.

Other effects of peroxisome proliferators, which may be important in hepatocarcinogenesis, include effects on transforming growth factor-β1 (which can initiate apoptosis) gene expression, inhibition of intercellular communication, modulation of intracellular calcium concentrations and the stimulation of protein kinase C [9].

6. Species differences

Marked species differences in response to rodent peroxisome proliferators have been reported [1,5,7–9]. When assessing species differences in response a number of factors should be considered. These include the metabolism, disposition and dose of the test compound, sex differences, as well as intrahepatic differences in response. Based on both marker enzyme activities and ultrastructural examination the rat and mouse are clearly responsive species, the Syrian hamster appears to exhibit an intermediate response, whereas in most studies the guinea pig is either non-responsive or refractory [1,5,7–9]. Generally, in vitro studies with primary hepatocyte cultures from the rat, mouse, Syrian hamster and guinea pig have supported the results of in vivo studies in these species [3,5,7–9,12].

Several studies have examined the ability of rodent peroxisome proliferators to produce effects in primates and humans. With respect to primates, studies with a number of compounds in both New (e.g. marmoset) and Old (e.g. Rhesus monkey) World monkeys have failed to provide any evidence of significant hepatic peroxisome proliferation [7,9]. However, albeit at high doses, 2 compounds, namely ciprofibrate and DL-040, have been reported to produce hepatic peroxisome proliferation in Cynomolgus and/or Rhesus monkeys [9]. Generally, peroxisome proliferators have not been reported to produce any significant effects (Table 3) on marker enzyme activities and/or peroxisomes in cultured primate hepatocytes [7–9,12].

Studies in humans have been conducted in patients treated with several hypolipidaemic agents (all being rodent peroxisome proliferators) including ciprofibrate, clofibrate, fenofibrate and gemfibrozil [7,8]. While most studies have failed to detect any significant changes, clofibrate was reported to produce a small increase in the number of peroxisomes and ciprofibrate to produce a small increase in the proportion of the hepatocyte cytoplasm occupied by peroxisomes. However, owing to the large interindividual variation in peroxisome morphometrics observed in these studies, together

Table 3
Species differences in the effects of peroxisome proliferators in hepatocyte cultures

Compound	Species[a]			
	Rat	Marmoset	Cynomolgus/ Rhesus monkey	Human
Beclobric acid	+[b]	n.d.	–	–
Benzbromarone	+	n.d.	n.d.	–
Ciprofibrate	+	n.d.	–	–
Clofibric acid	+	n.d.	–	–
Fomesafen	+	–	n.d.	–
Mono-(2-ethylhexyl)phthalate and derivatives	+	–	n.d.	–
Methylclofenapate	+	–	n.d.	–
Trichloroacetic acid	+	n.d.	n.d.	–

[a] Effect assessed by changes in enzyme activities and/or organelle proliferation.
[b] +, positive response; –, no/little response; n.d., not determined.

with cell to cell variations and lobular variations, it is difficult to attach any clear biological significance to these findings [7,8]. The effect of a number of rodent peroxisome proliferators on marker enzyme activities and/or peroxisomes has been studied in human hepatocytes [7–9,12]. Some examples are shown in Table 3. While such compounds produce significant concentration-dependent effects in rodent hepatocytes, they did not produce such effects in human hepatocytes [7–9]. Moreover, in many studies with primate and human hepatocytes, where peroxisome proliferators failed to produce any significant effects, the functional viability of the hepatocyte preparations was confirmed by parallel experiments in which effects on other endpoints were determined (e.g. stimulation of monooxygenase enzymes and tyrosine aminotransferase).

Some studies have also examined species differences in effects on cell replication. Both nafenopin and Wy-14,643 have been reported to stimulate replicative DNA synthesis in rat, but not in Syrian hamster, hepatocytes [9,25]. Although peroxisome proliferators can stimulate DNA synthesis in cultured rat hepatocytes, methylclofenapate was reported to be ineffective in guinea pig, marmoset and human hepatocytes and nafenopin has also been reported not to induce replicative DNA synthesis in human hepatocytes [7,9,26,27].

While many studies have demonstrated that the prolonged administration of peroxisome proliferators can produce liver tumours in the rat and/or mouse, few studies have examined the chronic effects of such compounds in other species. With the Syrian hamster clobuzarit did not produce tumours after 2 years [28] and nafenopin and Wy-14,643 did not produce tumours after 80 weeks administration [25]. In the marmoset clofibrate did not produce any tumours in a $6\frac{1}{2}$ year study, which is around half of the expected life-span of this species [29]. In other primate studies with compounds including ciprofibrate, clobuzarit and clofibrate, some increases in liver weight have been observed in some investigations, but no evidence for significant peroxisome proliferation or peroxisome proliferator-induced lesions has been reported [28–30].

7. Conclusions and risk assessment of rodent peroxisome proliferators

The key issues concerning the risk assessment of rodent liver peroxisome proliferators include:

(1) genotoxicity;
(2) compound potency – dose-response relationships, – no adverse effect levels;
(3) precise mechanism(s) of liver tumour formation;
(4) species differences in response – peroxisome proliferation, – tumour formation;
(5) likely human exposure.

Generally, peroxisome proliferators are considered to be non-genotoxic agents [6–9,17] and hence should be assessed differently from genotoxic carcinogens [31].

Rodent liver peroxisome proliferators exhibit clear no adverse effect levels for both peroxisome proliferation and for tumour formation. For example, in the rat no adverse effect levels for liver tumour formation have been observed in studies with several compounds including bezafibrate, clofibrate, di-(2-ethylhexyl)adipate (DEHA) and DEHP [6,9]. Moreover, the observed dose threshold for tumour formation in rodents is appreciably greater than the threshold for peroxisome proliferation and other effects [6,7,9].

Several mechanisms have been proposed to account for why peroxisome proliferators produce tumours in rodent liver. If these various hypotheses are combined then a role for increased cell replication in peroxisome proliferator-induced hepatocarcinogenesis may be readily identified. For example, if hepatocytes are transformed by either oxidative stress-induced damage or by alternative mechanisms, such initiated cells may be promoted to liver tumours by enhanced cell replication. Certainly peroxisome proliferators are effective promoters of certain populations of initiated cells and recent

studies suggest that peroxisome proliferators can influence rates of both cell replication and cell death in particular populations of hepatocytes [9,18,22,24,32]. Whatever the precise mechanism(s) of peroxisome proliferator-induced liver tumour formation in rodents, both peroxisome proliferation (i.e. organelle proliferation and associated marker enzyme activities) and cell replication are clearly important biomarkers.

In terms of species differences, rats and mice are clearly responsive species, whereas the majority of both in vivo and in vitro studies suggest that primates including man are either essentially refractory or certainly much less responsive to rodent peroxisome proliferators (Table 4). However, while effects on peroxisome morphology and marker enzyme activities have been extensively studied, few investigations have examined species differences in peroxisome proliferator-induced cell replication and liver tumour formation. Rodent peroxisome proliferators do not appear to stimulate replicative DNA synthesis in vivo in Syrian hamster hepatocytes and in vitro in human hepatocytes (Table 4). Moreover, while the clonal expansion of rat and mouse hepatocytes treated with both nafenopin and epidermal growth factor in soft agar could be observed, no such response was seen with Syrian hamster, guinea pig and human hepatocytes [33]. The limited data on the chronic effects of peroxisome proliferators in the Syrian hamster and primates suggests that, unlike the rat and mouse, liver lesions and tumours may not be formed.

Human exposure to rodent peroxisome proliferators depends on the intended usage of the particular compound. While hypolipidaemic agents are only administered to a restricted population of humans, exposure to industrial chemicals such as plasticizers is obviously far more widespread. In summary, the present literature suggests that exposure to rodent peroxisome proliferators as a class of chemicals does not pose any serious risk for man. Such compounds exhibit clear species differences in response (Table 4). Indeed apart from organelle proliferation and enzyme induction, the inability of peroxisome proliferators to stimulate replicative DNA synthesis in species other than the rat and mouse may be very important for risk assessment. However, it would be desirable to elucidate further the mechanism(s) of peroxisome proliferator-induced hepatocarcinogenesis in susceptible species (i.e. the rat and mouse). For example, it would be of interest to ascertain whether peroxisome proliferators could produce tumours in mutant mice which lack mPPARα [15]. Additional studies in rodents may help identify further biomarkers of tumour formation for use in studies of species differences including the use of human hepatocytes. Finally, further car-

Table 4
Species differences in the in vivo and in vitro effects of peroxisome proliferators

Species	Test system[a]	Parameter		
		Organelle proliferation	Enzyme induction[b]	Cell replication
Rat and mouse	In vivo	+[c]	+	+
	In vitro	+	+	+
Syrian hamster	In vivo	+	+	−
	In vitro	?	+	?
Guinea pig	In vivo	−	±	−
	In vitro	?	−	?
Human	In vivo	±	?	?
	In vitro	−	−	−

[a] In vivo refers to studies in intact animals or in human subjects receiving hypolipidaemic agents. In vitro refers to studies in cultured hepatocytes.
[b] Enzyme activities include palmitoyl-CoA oxidation, lauric acid 12-hydroxylase and carnitine acetyltransferase.
[c] +, positive response; ±, equivocal response and/or literature data; −, no/little response; ?, not studied.

cinogenicity studies in partially responsive (e.g. Syrian hamster) and non-responsive (e.g. guinea pig) species would strengthen the conclusion that exposure to peroxisome proliferators do not constitute any significant risk to man.

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Oxidative stress in nongenotoxic carcinogenesis

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Abstract

The induction of oxidative stress in the target tissue has been proposed as a possible mechanism of action for nongenotoxic carcinogens. A variety of nongenotoxic hepatocarcinogens including peroxisome proliferators, organochlorines, barbiturates, and metals have been shown to produce an increase in reactive oxygen species (ROS) in the liver. Our group has examined the induction of oxidative stress by the organochlorine mouse hepatic carcinogen, dieldrin. Using a salicylate spin trap assay, dieldrin was found to produce mouse liver-specific increases in ROS in cultured hepatocytes. Increased amounts of hepatic 8-hydroxy-2′-deoxyguanosine and malondialdehyde (MDA) and decreased levels of cellular antioxidants were also seen in cultured mouse hepatocytes following dieldrin treatment. In subchronically dieldrin-treated mice and rats, hepatic vitamin E (Vit E) was decreased correlated with dieldrin dose. While Vit E levels were decreased in both rats and mice, the normal lower levels of Vit E in the mouse resulted in a subsequent oxidative stress, evidenced by an increase in MDA formation in the mouse liver. Dieldrin also produced a dose-dependent increase in DNA synthesis in the mouse (not the rat) following subchronic treatment. These effects seen in both cells in culture and in vivo were species specific, organ specific, and dose dependent which directly correlated with the observed pattern of cancer induction for dieldrin in rodents (mouse liver-specific). These findings support a possible role for the induction of oxidative stress in nongenotoxic hepatic carcinogenesis possibly through modulation of gene expression.

Keywords: Oxidative stress; Nongenotoxic carcinogenesis; Dieldrin; Vitamin E

1. Introduction

The formation of reactive oxygen species (ROS) and subsequent induction of oxidative stress by xenobiotics has been proposed as a mechanism for chemically induced carcinogenesis [1–4]. Hydroxylation of DNA and the subsequent production of initiated cells by reactive free radicals has been suggested for the initiation step in carcinogenesis but the mechanisms of tumor promotion by ROS producing xenobiotics remains unclear. Hadler et al. [5] has suggested that mitochondrial mutations may lead to a modified growth advantage for initiated cells. Solt and Farber [6] introduced a broader definition in which promotion occurs when initiated hepatocytes are resistant to the toxic effects of xenobiotics giving them a selective growth ad-

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vantage over normal cells. Cell proliferation or inhibition of apoptosis in initiated cells has also been proposed as a mechanism of ROS-generated carcinogenesis by xenobiotics.

Many chlorinated compounds selectively induce hepatic cancer in mice but not rats. Dieldrin is an example of one such compound. Initial studies have shown dieldrin to induce centrilobular DNA synthesis in the B6C3F1 mouse but not the rat [7]. This phenomenon was partially reduced by the addition of 200 ppm vitamin E (Vit E) to the diet. These findings suggested a role for oxidative stress in the selective hepatoxicity and possible carcinogenicity of dieldrin. The present study further examined the possible role of ROS generation and oxidative stress formation in dieldrin hepatocarcinogenesis.

Oxidative stress has been defined as "a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage" [8]. Mammalian cells generate ROS during normal metabolic processes. The cell has several ways to respond to the production of ROS by either directly reducing the ROS via scavenging by enzymatic and non-enzymatic antioxidants or by removing the oxidative damage by-products. ROS consist of reduced forms of diatomic oxygen including superoxide, hydrogen peroxide and the hydroxyl radical. Superoxide, the least reactive of these, cannot pass through cellular membranes but it can be spontaneously or enzymatically dismutased to hydrogen peroxide [9]. Hydrogen peroxide is electrically neutral and can pass through cellular membranes. It is typically present in cells at concentrations of 10^{-7}–10^{-9} M. Its conversion to the more reactive hydroxyl radical occurs either by the Haber-Weiss or the Fenton reactions [10,11]. The hydroxyl radical, the most reactive of the ROS causes cellular damage in the immediate vicinity of its generation [12].

Hepatocytes live in a balance of free radical production, free radical scavengers and repair of damage caused by free radicals. The addition of xenobiotics to hepatocytes can upset this balance by: (1) directly forming free radicals; (2) indirectly forming ROS from within the cell (through cytochrome P450, peroxisomes or mitochondria); (3) depleting or inhibiting normal enzymatic and non-enzymatic antioxidant systems which scavenge the free radicals and protect the cell; or (4) overburdening the scavenger and repair mechanisms in the cell.

Cells use oxygen in many physiological processes. Peroxisomes, which break down fatty acids, generate hydrogen peroxide in the process. Catalase located in the peroxisomes can use this peroxide in detoxification reactions to metabolize compounds such as formaldehyde, alcohol and formic acid [13,14]. Likewise, mitochondria generates ROS during the cellular respiratory cycle and fatty acid catabolism [15]. Mn superoxide dismutase, as well as other antioxidants within the mitochondria, maintain the spurious ROS generated by this process at low levels. Work by Sohal and coworkers has in fact demonstrated a strong inverse correlation between production of ROS by mitochondria and longevity of mammalian species [16,17]. A third important site of free radical generation, the microsomal electron transport system (cytochrome P450), requires electrons from NADPH to produce partially reduced oxygen species. Normally, this process does not result in production of ROS but in the presence of selected xenobiotics; superoxide radicals may be generated through futile cycling [18]. The superoxide radical can then be converted to the more reactive hydroxyl radical via the Haber-Weiss reaction.

In addition to the intracellular production of ROS, extracellular events can also mediate oxidative stress. Macrophages generated ROS when activated to begin phagocytosis. Macrophages undergo oxidative burst which are catalyzed by a membrane-bound enzyme NADPH oxidase [19]. An oxidative burst is characterized by the release of large amounts of superoxide from the macrophages. Macrophages can be activated by xenobiotics such as carbon tetrachloride and dieldrin [20,21]. The activated Kupffer cells can also increase production of tumor necrosis factor alpha (TNFα) which triggers the conversion of arginine to citrulline releasing nitric oxide (NO) via NO synthetase [22]. Furthermore, hypochlorite (HOCl) can be produced by myeloperoxidase (MPO) [10]. The NO

radical, itself, can react with oxygen to produce peroxynitrite, an intermediate capable of decomposing into the more active peroxide radical [9].

The antioxidant defense systems of the hepatocyte can be divided into non-enzymatic and enzymatic. The non-enzymatic antioxidant defenses consist of molecules such as uric acid (UA), Vit E, vitamin C (Vit C) and glutathione (GSH) that can directly act on free radicals. The enzymatic antioxidant defenses consist of superoxide dismutase (SOD), catalase (CAT), GSH peroxidase (GSH-Px) and GSH *S*-transferase (GST).

The enzymatic defense systems consist of substances that either directly act to remove free radicals or act to recycle non-enzymatic molecules. The cornerstones of the enzymatic system are 3 enzymes: SOD, CAT and GSH-Px. SOD takes the superoxide radical and converts it to hydrogen peroxide [23,24]. SOD and CAT have been shown to decrease in skin upon treatment with the tumor promoter TPA [25]. Also, SOD and GSH-Px have been shown to decrease in hepatocellular carcinomas [26]. Thus, changes in cellular enzyme levels may give initiated cells a selective growth advantage.

In the present study the ability of dieldrin to induce ROS (as measured by salicylate spin trap), oxidative damage (as measured by 8-hydroxy-2′-deoxyguanosine (oh8dG) and lipid peroxidation) and modulate antioxidatant (measured by changes in Vit E) was examined. Previous studies by our group have shown that the enzymatic antioxidants do not change with dieldrin treatment and that other non-enzymatic antioxidants are modified to the same extent as Vit E. The current studies were performed in primary cultured hepatocytes from rat and mouse and in subchronically diedrin-treated rats and mice.

2. Materials and methods

Mouse and rat hepatocytes from F344 rats and B6C3F1 mice (Harlan-Sprague Dawley, Inc., Indianapolis, IN) were isolated by in situ portal vein perfusion according to Klauning et al. and Klaunig and Baker [27,28]. Isolated cells were plated at a density of 33 000 cells/cm^2 onto either 60-mm culture dishes in DMEM/F12 media supplemented with insulin (5 μg/ml), gentamycin sulfate (50 mg/ml), dexamethasone (0.8 μg/ml: stock solution 4 mg/ml 95% ethanol) and 5% fetal bovine serum. Cells were incubated in a 5% carbon dioxide incubator set at 37°C and 95% humidity. Medium was changed after 4 h and every 24 h thereafter. Cells were dosed with dieldrin at each medium change. To determine if dieldrin generates ROS, dieldrin-treated B6C3F1 and F344 cultured hepatocytes and the spin trap method of Floyd et al. [29] was used as modified by Grootveld and Halliwell [30]. Oxidative damage (as measured by oh8dG and lipid peroxidation) and modulated antioxidant (measured by changes in Vit E) was examined in both cultured hepatocytes and liver from rats and mice treated with dieldrin. Lipid peroxidation (thio-barbituric acid reactive species (TBARS)) was determined by the method of Uchiyama and Mihara [31]. oh8dG was determined by a modified procedure of Marmur [32] as described by Chung and Xu [33]. Vit E was measured using HPLC-UV detection.

3. Results

Hepatocytes were incubated with salicylic acid and dosed with dieldrin (0.1, 1, 5, 10, 25, and 50 μM) for 60 min. Mouse hepatocytes dosed with 25 μM and 50 μM dieldrin resulted in a 2-fold increase in 2,3-dihyroxybenzoic acid (2,3-DHBA) formation (Table 1). Hepatocytes isolated from mice pretreated for 14 days with dieldrin showed a similar increase in 2,3-DHBA production (2.5 times control hepatocytes). Rat hepatocytes, normal or dieldrin-pretreated (induced) showed no increase in 2,3-DHBA (Table 1).

Mouse and rat hepatocytes were treated for 24 h with 0.1, 1, 5, 10, 25 and 50 μM dieldrin to measure malonedialdehyde (MDA) levels (Table 2). An increase was observed in mouse hepatocytes at concentrations of 25 and 50 μM dieldrin. No change was observed in rat hepatocytes.

Table 1
2,3-Dihydroxybenzoic acid in mouse and rat hepatocytes following dieldrin treatment

Dieldrin concentration (μM)	Normal mouse	Normal rat	Treated mouse	Treated rat
0	0.065	0.133	0.047	0.040
0.1	0.068	0.158	0.046	0.061
1.0	0.096*	0.138	0.049	0.071
5.0	0.090*	0.093	0.061*	0.027
10.0	0.095*	0.085	0.082*	0.047
25.0	0.140*	0.082	0.118*	0.037
50.0	0.136*	0.093	0.113*	0.040

Values are the mean ±S.D. based on 3 samples per group. Mice and rats were 6–8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin for 2 weeks prior to hepatocyte isolation. Statistical analysis was accomplished by first using ANOVA with $P < 0.05$ followed by the post hoc Student t-test. *Significant $P < 0.05$ when compared to control. Values are expressed as (2, 3-DHBA/SA) × 100.

Table 2
Vit E, MDA and oh8dG levels for B6C3F1 mouse- and F344 rat-cultured hepatocytes following dieldrin treatment

Dieldrin concentration (μM)	Vit E (pmol/mg protein)		MDA (nmol/mg protein)		oh8dG/dGuo × 10^{-5}	
	Mouse	Rat	Mouse	Rat	Mouse	Rat
0	11.10 ± 0.81	21.54 ± 1.83	0.54 ± 0.022	0.49 ± 0.06	6.96 ± 0.37	2.75 ± 1.24
0.1	10.60 ± 1.99	20.25 ± 0.72	0.54 ± 0.042	0.58 ± 0.06	7.05 ± 1.50	3.53 ± 1.61
1.0	9.31 ± 2.50*	11.85 ± 0.89*	0.55 ± 0.037	0.61 ± 0.08	8.94 ± 2.05	4.08 ± 2.33
5.0	7.81 ± 1.89*	9.23 ± 0.34*	0.53 ± 0.084	0.67 ± 0.08	8.92 ± 1.80	3.26 ± 1.31
10.0	4.45 ± 1.10*	6.88 ± 0.48*	0.62 ± 0.075	0.60 ± 0.11	12.93 ± 1.54*	3.69 ± 1.32
25.0	3.31 ± 0.84*	4.88 ± 0.34*	0.76 ± 0.154*	0.48 ± 0.09	26.44 ± 9.99*	4.72 ± 1.57
50.0	2.40 ± 1.33*	6.00 ± 0.73*	0.96 ± 0.105*	0.55 ± 0.09	9.8 ± 2.96	3.73 ± 0.63

Values are the mean ±S.D. based on 3 samples per group. Mice and rats were 6–8 weeks of age. Animals were placed on diets of NIH-07 for 1 week prior to hepatocyte isolation. Hepatocytes were treated for 24 h before analysis. Statistical analysis was accomplished by first using ANOVA with $P < 0.05$ followed by the post hoc Student t-test. *Significant $P < 0.05$ when compared to control.

A significant increase in oh8dG was observed only in mouse hepatocytes following treatment with dieldrin concentrations of 10 and 25 μM (Table 2). Measurement of antioxidant levels in cultured hepatocytes following dieldrin treatment showed a dose-dependent decrease in Vit E in both mouse and rat hepatocytes. While Vit E decreased to about 20% of their original values in both rats and mice, the rat initially contained about twice the amount of Vit E as that seen in the mouse (Table 2).

Dieldrin was administered in the diet to B6C3F1 mice and F344 rats. Rats and mice treated with dieldrin (0.0, 0.1, 1.0, and 10.0 mg/kg diet) were sacrificed after 7, 14, 28, and 90 days of exposure. Hepatic DNA synthesis as measured by autoradiography showed a dieldrin dose-related increase that was restricted to the treated mice (Table 3). No increase in DNA synthesis was seen in the rat. Mice showed an increase in DNA synthetic labelling after 7 days to 4.4% at 10 mg/kg and 2.6% at 1.0mg/kg. The labelling index appeared to pleateau after 14 days of exposure and returned to near basal levels by day 90. At all time points the 10-mg/kg dieldrin diet produced a significant increase above controls in mice.

In these same subchronically treated rodents, both the mouse and the rat showed a significant decrease in Vit E levels with dieldrin (Table 4). Both species showed an initial 50% decrease in hepatic Vit E levels with dieldrin but only the mouse showed a clean dose response on days 7 and 14 which correlated with dietary dieldrin. The rat control hepatic Vit E was approximately 3 times that of the mouse. This may afford the

Table 3
Hepatic DNA S-Phase synthesis for B6C3F1 mice and F344 rats in subchronic dieldrin study

Dieldrin (mg/kg diet)	Sampling time (days)	DNA S-Phase synthesis (% labeled nuclei)	
		Mouse	Rat
0.0	7	1.54 ± 0.50	1.84 ± 0.77
0.1	7	1.42 ± 0.52	1.28 ± 0.32
1.0	7	2.57 ± 0.84*	2.20 ± 0.97
10.0	7	4.43 ± 1.14*	1.17 ± 1.21
0.0	14	1.17 ± 0.34	1.06 ± 0.35
0.1	14	1.66 ± 0.62	1.26 ± 0.51
1.0	14	2.35 ± 0.42*	1.11 ± 0.31
10.0	14	10.72 ± 3.38*	1.06 ± 0.24
0.0	28	2.55 ± 0.52	1.89 ± 0.49
0.1	28	2.83 ± 0.88	2.66 ± 0.47
1.0	28	4.44 ± 1.25*	2.10 ± 0.20
10.0	28	10.41 ± 2.44*	4.65 ± 1.15
0.0	90	1.89 ± 0.49	1.31 ± 0.43
0.1	90	2.66 ± 0.47*	1.31 ± 0.43
1.0	90	2.10 ± 0.20	1.39 ± 0.31
10.0	90	4.65 ± 1.16*	1.61 ± 10.38

Five animals were used per dose group per time period. Results are expressed as ±S.D. Statistical analysis was accomplished using ANOVA ($P < 0.05$ criteria) followed by the post hoc Student t-test. *Value significantly different from that of control (no dieldrin).

Table 4
Hepatic Vit E levels for B6C3F1 mice and F344 rats following subchronic dieldrin treatment

Dieldrin (mg/kg diet)	Sampling time (days)	Hepatic Vit E (μmmol/g tissue)	
		Mouse	Rat
0.0	7	0.99 ± 0.04	3.63 ± 0.52
0.1	7	0.76 ± 0.12*	1.83 ± 0.63*
1.0	7	0.67 ± 0.04*	1.83 ± 0.47*
10.0	7	0.49 ± 0.15*	1.82 ± 0.66*
0.0	14	1.08 ± 0.06	3.62 ± 0.52
0.1	14	0.76 ± 0.09*	2.79 ± 0.28*
1.0	14	0.71 ± 0.07*	2.67 ± 0.30*
10.0	14	0.60 ± 0.12*	2.63 ± 0.49*
0.0	28	1.16 ± 0.14	3.44 ± 0.51
0.1	28	0.85 ± 0.08*	2.92 ± 0.18*
1.0	28	0.77 ± 0.05*	3.15 ± 0.27
10.0	28	0.87 ± 0.08*	2.55 ± 0.41
0.0	90	1.04 ± 0.09	3.19 ± 0.15
0.1	90	0.65 ± 0.05*	1.99 ± 0.26*
1.0	90	0.53 ± 0.05*	2.12 ± 0.16*
10.0	90	0.47 ± 0.04*	2.24 ± 0.26*

Five animals were used per dose group per time period. Results are expressed as ±S.D. Statistical analysis was accomplished using ANOVA ($P < 0.05$ criteria) followed by the post hoc Student t-test. *Value significantly different from that of control (no dieldrin).

rat better protection against ROS. Hepatic MDA showed an initial increase in the mouse liver at days 7 and 14 which correlated to dietary dieldrin. No change in liver MDA was observed in the rat (Table 5). No change was found in oh8dG (a useful marker for oxidative damage to DNA) in mouse and rat livers treated with dieldrin (Table 6).

Table 5
Hepatic MDA in B6C3F1 mice and F344 rats following subchronic dieldrin treatment

Dieldrin (mg/kg diet)	Sampling time (days)	Hepatic MDA (nmol/g tissue)	
		Mouse	Rat
0.0	7	14.50	11.37
0.1	7	29.70*	15.77
1.0	7	39.76*	11.99
10.0	7	45.00*	11.41
0.0	14	25.00	12.94
0.1	14	54.00*	11.69
1.0	14	47.00*	11.11
10.0	14	83.60*	11.81
0.0	28	25.00	11.37
0.1	28	39.40*	15.77
1.0	28	48.80*	11.99
10.0	28	38.50*	11.41
0.0	90	30.80	11.65
0.1	90	37.40	9.12
1.0	90	31.80	9.96
10.0	90	36.20	8.64

Results are expressed as ±S.D. Statistical analysis was accomplished using ANOVA ($P < 0.05$ criteria) followed by the post hoc Student t-test. *Value significantly different from that of control (no dieldrin).

Table 6
Hepatic oh8dG in B6C3F1 mice and F344 rats following subchronic dieldrin treatment

Dieldrin (mg/kg diet)	Sampling time (days)	Hepatic oh8dG (oh8dG/dGuo $\times 10^{-5}$)	
		Mouse	Rat
0.0	7	5.00	3.04
0.1	7	6.28	1.96
1.0	7	4.42	3.80
10.0	7	6.01	3.59
0.0	14	4.36	3.77
0.1	14	3.26	3.38
1.0	14	2.89	3.69
10.0	14	3.18	3.35
0.0	28	4.26	3.94
0.1	28	5.36	2.55
1.0	28	5.43	3.46
10.0	28	3.38	2.79
0.0	90	5.44	2.85
0.1	90	5.59	3.29
1.0	90	4.97	3.02
10.0	90	5.70	2.50

Results are expressed as ±S.D. Statistical analysis was accomplished using ANOVA ($P < 0.05$ criteria) followed by the post hoc Student t-test. *Value significantly different from that of control (no dieldrin).

4. Discussion

The detection of ROS by salicylate was adapted in this study for the detection of ROS in primary hepatocytes. Salicylate can form several adducts on exposure to ROS, but only the 2,3-DHBA is exclusively produced by this method. The other common adduct, 2,5-dihyroxybenzoic

acid (2,5-DHBA), can be generated by direct metabolism of salicylic acid via the P450 system. A change in the level of the 2,5-DHBA might reflect either competition for the P450 isoenzyme metabolizing salicylate or a possible increase in its metabolism by the interaction of another substance with cytochrome P450. The fact that only mouse liver produce free radicals supports the results showing an increase in oh8dG and MDA levels following dieldrin treatment in mouse, but not rat hepatocytes.

In subchronicallly treated rodents, the mouse also shows unique effects of dieldrin treatment when viewed in light of the antioxidant/pro-oxidant balance. While both the rat and mouse showed a decrease in hepatic Vit E only the mouse showed signs of hepatic oxidative stress as evidenced by increased hepatic MDA. The stimulation of DNA synthesis observed in the mouse paralleled the increase in hepatic MDA and the increase in urinary oh8dG, suggesting that the increase in DNA synthesis may be related to the reduction of oxidative stress.

Stimulation of DNA synthesis in subchronic studies is a predictive tool to evaluate the carcinogenicity of xenobiotics [34]. Other investigators have reviewed the connection between cell proliferation and cancer [35,36]. Cancer by definition is a proliferative disease and DNA synthesis is critical to all stages of cancer. Cell proliferation is required to fix a mutation which occurs in an initiated cell and the clonal expansion of these cells during promotion cannot occur without cell proliferation. Furthermore, when cells are in S phase they are more vulnerable to the genotoxic effects of chemicals since the time for DNA repair is limited and the DNA is more exposed [37]. oh8dG levels rose in the urine in relation to the increase in DNA S-phase synthesis suggesting that the DNA was more exposed to the effects of oxidative stress at this time (data not shown). Thus, stimulation of DNA synthesis prior to formation of preneoplastic foci may allow for the fixation of spontaneously occuring mutations or mutations resulting from genotoxic compounds.

The increase in hepatic MDA in the mouse preceded the observed increase in DNA synthesis. MDA, a byproduct formed during free radical damage of polyunsaturated fatty acids (PUFA), has been shown to be mutagenic to bacteria and mammalian cells and carcinogenic to rats [38,39]. MDA is capable of forming adducts with DNA [40]. The decrease in MDA in dieldrin-treated mouse livers to control levels at later time points may be due to either improved removal of MDA from the liver or an increase in the antioxidant balance of the liver. Surprisingly, an increase in mouse hepatic oh8dG levels was not observed. This is in contrast to that seen in the cultured hepatocytes. While total oh8dG was not modified, other studies have suggested that mitochondrial oh8dG (in contrast to nuclear DNA damage) may be the target for the damage by xenobiotics. Current ongoing studies in our group are further examining the role of mitochondrial damage in this process.

At early time points we observed a dose-dependent decrease in hepatic Vit E levels in the mouse which correlated with the observed increase in MDA. Later, the dose response could not be observed possibly due to bio-accumulation of dieldrin in the liver. The decrease in Vit E may be due to its displacement from the membrane, oxidation by free radicals or interference with its transport in the body.

Oxidative stress may cause an increase in DNA S-phase synthesis through a variety of mechanisms. Vit E has been shown to inhibit PKC activity which is involved in many cellular proliferation pathways [41]. The decrease in hepatic Vit E observed in this study then might explain the proliferative event. Activation of oncogenes such as AP-1 or NF-kB by oxidative stress [42] might result in an increase in DNA synthesis. Oxidative stress has been shown to cause release of calcium ions from cellular stores which may in turn lead to mitotic events [43].

Oxidative stress is a pervasive phenomenon resulting from the in vitro or in vivo treatment of hepatocytes with tumor promoting xenobiotics. These xenobiotics may act through a variety of different mechanisms (i.e. peroxisome proliferation, direct production of free radicals by the cytochrome P450 system or by altering the antioxidant defense system of the cell). This re-

search focused on the selective action of dieldrin in the mouse liver prior to the appearance of preneoplastic foci and tumors. The question arises as to how this oxidative stress observed with dieldrin treatment correlates to hepatocarcinogenicity observed in the mouse. Several possibilities exist which focus on nongenotoxic mechanisms of cancer, including the interrelationship between oxidative stress, aging and cancer, modulation of redox regulation of transcription factors and lastly, a change in mitochondrial damage.

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Clofibrate-induced neoplastic development in the rat liver is associated with decreased connexin 32 expression but not with a co-ordinated shift in expression of marker enzymes

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Abstract

Altered enzyme phenotype and expression of connexin 32 (Cx32), a gap junction protein were studied during the development of rat liver tumors induced by the non-genotoxic carcinogen, clofibrate. (1) In contrast to previous findings for nitrosamine-induced lesions, preneoplastic enzyme-altered foci (EAF) and neoplastic nodules (NN) lacked any clear association with degree of altered enzyme expression because of an almost complete negativity for GST-P and GGT. (2) Immunohistochemically demonstrated Cx32 spots on the hepatocyte membranes showed a clear decrease in clofibrate-induced lesions. (3) Naturally occurring EAF demonstrating GST-P and/or GGT positivity did not show a significant decrease of Cx32 counts suggesting a reversible nature. Therefore, the Cx32 decrease appears closely linked to progression of hepatocarcinogenesis irrespective of the enzyme phenotype of neoplastic focal lesions and the carcinogens used for their induction.

Keywords: Clofibrate; Connexin 32; Enzyme; Liver tumor; Rat

1. Introduction

Preneoplastic lesions exhibit various enzyme alterations such as decreased glucose-6-phosphatase (G6Pase) and adenosine triphosphatase (ATPase), and increased γ-glutamyl transpeptidase (GGT), glucose-6-phosphate dehydrogenase (G6PD) and the glutathione *S*-transferase placental form (GST-P) [1–5], all of which have been used as markers for lesion development. We have reported that the degree of phenotypic changes in terms of numbers of such enzyme phenotypes shows a good correlation with neoplastic development from early EAF to hepatocellular carcinomas (HCC) [6].

Gap junctional intercellular communication (GJIC) maintained by connexin protein is known

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to play an important role in physiological homeostasis by mediating transfer of signal-transducting substances involved in control of metabolism and cell division [7]. Aberrant expression of GJIC has been described in neoplastic cells and its interruption shown to be directly relevant to failure of homeostatic control and resultant carcinogenesis [8,9]. In the rat liver, chemically induced preneoplastic and neoplastic lesions, such as EAF, NN and HCC, which are known to express various phenotypic changes, show obvious decreases in levels of liver gap junction connexin 32 (Cx32) mRNA and its immunohistochemically demonstrated protein [10–12]. With regard to the atypical lesions induced by peroxisome proliferators, a clear difference has been reported for altered enzyme status but it has hitherto not been clarified whether their capacity for cell-cell communication is impaired [13].

The present investigation was therefore conducted at assessing (1) Cx32 expression in preneoplastic lesions induced by clofibrate, a non-genotoxic carcinogen known as a peroxisome proliferator [13]; and (2) correlation of the degree of simultaneously expressed altered enzyme phenotypes within each lesion and progression of hepatocarcinogenesis. The results were compared with our previous findings for lesions induced by a genotoxic hepatocarcinogen, *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN).

2. Materials and methods

2.1. Animals and experimental schedule

Male 6-week-old Wistar rats (Charles River Japan Inc., Atsugi, Japan) were used. They were maintained on basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) ad libitum and housed in plastic cages in an air-conditioned room at 24 ± 2°C and 60 ± 5% humidity. For the induction of preneoplastic and neoplastic hepatocyte lesions, rats were placed on the basal diet containing 0.5% clofibrate for 104 weeks and then killed. Control animals were also killed at the same age.

2.2. Histochemistry and immunohistochemistry

Immediately upon sacrifice, the livers were excised and slices 4–5 mm thick were cut with a razor blade and immersed in isopentane precooled to approximately −130°C in a liquid nitrogen bath. They were stored at −80°C in a deep-freezer until use. Serial sections cut at 4 μm were used for the histochemical demonstration of G6PD and succinate dehydrogenase (SD) [14], and of G6Pase, ATPase and GGT activities [15]. For the immunohistochemical staining of GST-P and Cx32, frozen sections were fixed in acetone cooled to −20°C, before treatment with polyclonal antibody to GST-P [16] at a dilution of 1:5000, or monoclonal antibody to Cx32 [8] at a dilution of 1:600. Binding sites were demonstrated by the avidin-biotin peroxidase complex (ABC) method using diaminobenzidine-H_2O_2, and sections were then lightly counterstained with hematoxylin.

2.3. Enzyme expression class analysis

For the quantitative analysis of altered enzyme expression, individual lesions demonstrating the respective enzyme alteration, in more than half of the focal lesion area, were traced and overlaid on a sheet of paper using a Microfiche Plaque Viewer (Carl Zeiss, Jena, Germany) at the magnification of ×13. For each individual focal population assessed, phenotypic expression was noted. The lesions were classified into one of six different classes on the basis of numbers of the following five different enzyme alterations: (a) increase of GST-P; (b) increase of GGT; (c) decrease of ATPase; (d) decrease of G6Pase; (e) increase or decrease of G6PD; (f) decrease or increase of SD, as compared to surrounding hepatocytes. For example, class 3 lesions expressed three out of the above six enzyme alterations in any combination. The smallest EAF included were 0.2 mm in diameter. The criterion for distinguishing EAF from NN was the presence of obvious compression of the surrounding parenchyma in the latter lesions which, in general, exceed one liver lobule [17].

Ten clofibrate-induced EAF and NN (negative for both GST-P and GGT), as well as 74 GST-P- and GGT-positive EAF and naturally occurring EAF in untreated liver were included for the analysis.

2.4. Quantitative analysis of Cx32 localization

Counting of Cx32 spots was performed using a microscope equipped with a grid-mesh eye-piece lens, which allowed approximately 250–300 normal hepatocytes to be counted within one field at ×400 magnification. Cx32 values were calculated by counting the number of immunohistochemically demonstrated Cx32 spots (connexon) on hepatocyte membranes and expressed as the number/cell. For the comparison of Cx32 counts, 17 grid areas of background parenchyma in five clofibrate-treated, 13 grid areas of the 10 EAF, and nine grid areas from five NN were taken from five of the experimental group rats. As controls, 9 grid areas of background and 20 grid areas of 10 EAF from five untreated livers were used.

3. Results

3.1. Enzyme expression characteristics

Two different types of lesions, tentatively named Type I and Type II lesions, were observed in the livers of clofibrate-treated rats. Type I lesions, both NN and large size EAF, were characterized by a lack of both GST-P and GGT (no alteration compared to surrounding hepatocytes) (Fig. 1). This type of lesion expressed decreased ATPase and/or G6Pase activities as compared to surrounding hepatocytes, similar to EHEN-induced lesions. However, in most cases, expression of G6PD decreased and SD increased, which was in contrast to EHEN-induced lesions which expressed an increase in G6PD and unchanged or decreased SD.

Type II lesions, characterized by positive reactions for both GST-P and GGT or either one of them, were also found within the same liver sections. Most of these lesions were foci size and their enzyme phenotype was closely similar to that observed for EHEN-induced lesions. Numbers of Type I and II lesions in the livers of clofibrate-treated rats were 3.42 ± 1.63 and 14.33 ± 9.80 per cm^2, respectively.

In the untreated liver, appreciable numbers of 'spontaneously' occurring lesions were observed. They were GST-P and/or GGT positive and their expression of other enzymes was closely similar to that observed in Type II and EHEN-induced lesions. The number of this type of lesion was 11.96 ± 2.95 per cm^2, the value not being significantly different from that of Type II lesions in clofibrate-treated liver (Fig. 2).

3.2. Degree of enzyme expression class

Since Type I lesions lacked expression of GST-P and GGT, their degree of altered enzyme expression was limited to within the range of classes 2–4, with class 3 predominating (45%). In contrast, Type II lesions exhibited a broad distribution class through classes 1–6, with class 3 including the highest number (24.3%). In spontaneously-occurring lesions, the expression class broadly ranged from classes 1 to 6, as observed in Type II lesions, with class 2 being the largest (41.6%) (Fig. 3).

3.3. Quantitative analysis of Cx32 spots

In the liver of clofibrate-treated rats, Cx32 count/cell was 0.15 ± 0.12 in Type I lesions, 2.30 ± 0.59 in Type II lesions and 1.95 ± 0.41 in surrounding hepatocytes. In the liver of untreated rats, Cx32 count was 3.52 ± 0.82 in naturally occurring lesions and 3.71 ± 0.53 in surrounding hepatocytes. Cx32 count in Type I lesion was significantly decreased as compared to Type II lesions and surrounding hepatocytes (Fig. 4). Values of Type II and naturally occurring lesions were not significantly different from respective surrounding hepatocytes.

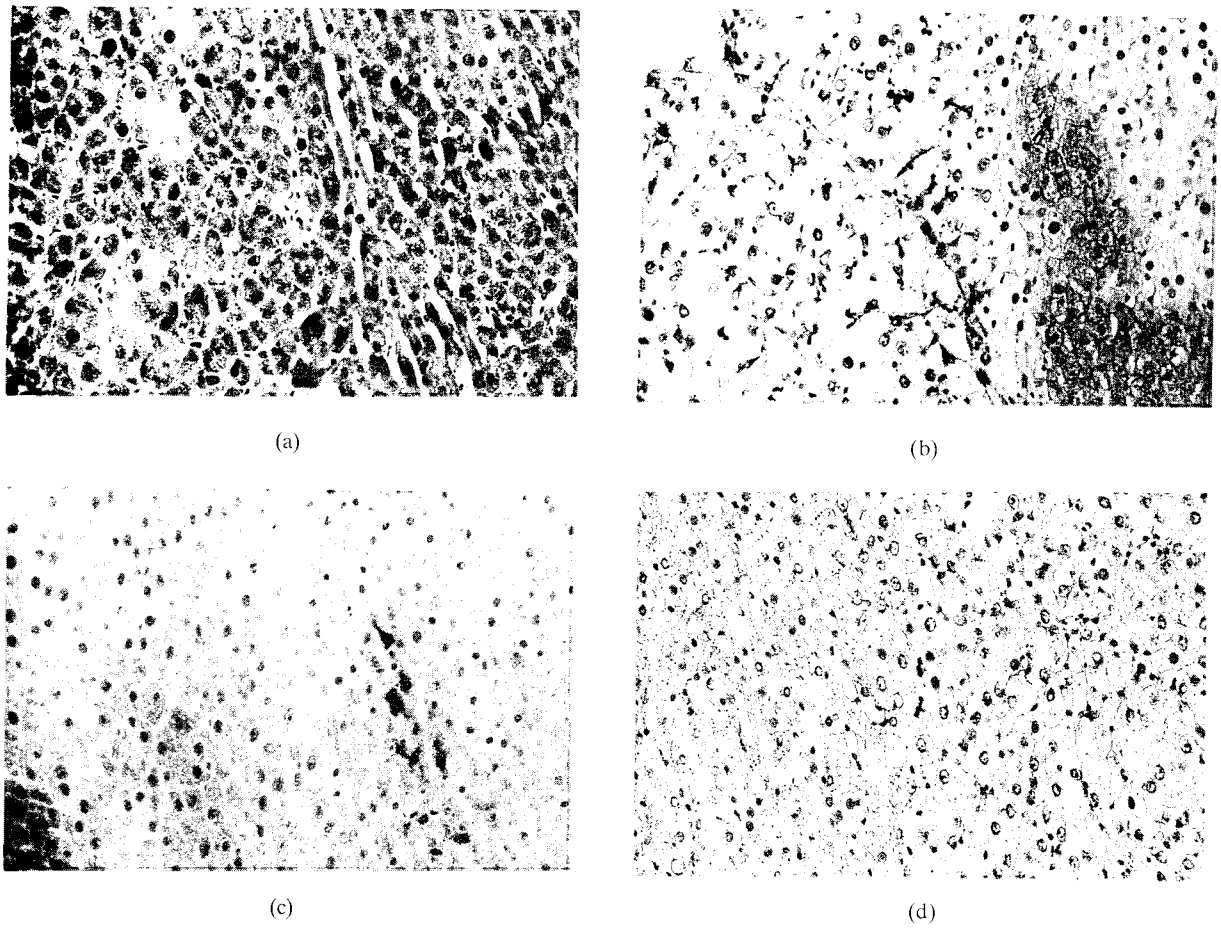


Fig. 1. A clofibrate-induced Type I lesion (left half) lacking GST-P, GGT and Cx32 spots. (a) H&E; (b) GST-P; (c) GGT; (d) Cx32. The GST-P and GGT positive area compressed outside the Type I lesion is a Type II focus in which Cx32 spots are not decreased.

4. Discussion

The rationale for the present study of class profile analysis of altered enzyme expression is based on our previous finding that preneoplastic lesions induced by EHEN exhibit a gradient shift toward larger expression class during the progression of neoplastic development. All transplanted HCC was found in the extreme class 5, despite no specific combination of altered enzymes being apparent among the different class lesions [6]. Thus, the degree of phenotypic shift is positively associated with progression toward malignancy. However, in contrast to EHEN-induced lesions, our present results clearly indicate that clofibrate-induced Type I lesions did not show a gradient shift toward larger numbers of enzyme expression class, mainly because they characteristically lack expression of two enzymes, GST-P and GGT, as previously observed [18,19]. In addition to the negative reactions for these two enzymes, G6PD was conspicuously decreased given the strong increase seen in these nitrosamine-induced lesions [5,6]. Further investigation is necessary to clarify the role of decrease in G6PD in terms of advantage for the neoplastic development.

The relative absence of small Type I lesions might be explained by a promotion by clofibrate of spontaneously-occurring foci together with

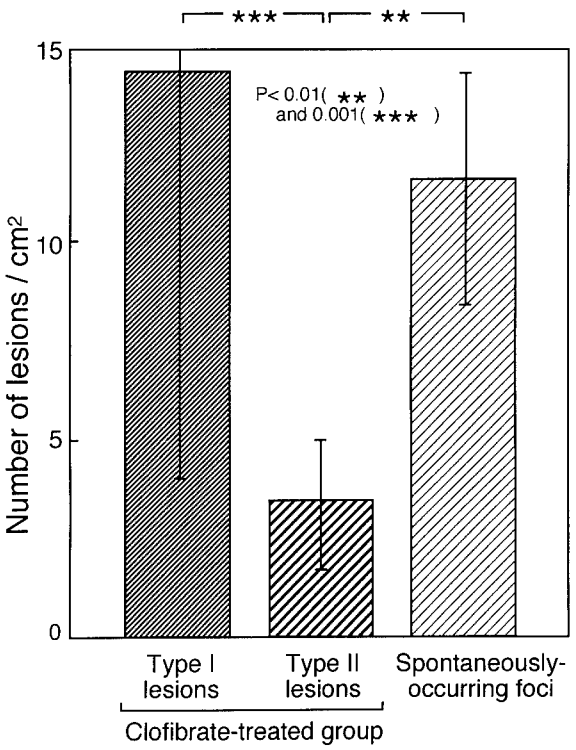


Fig. 2. Numbers of liver lesions in clofibrate-treated and untreated rats. No significant difference is evident between Type I lesions and spontaneously-occurring foci.

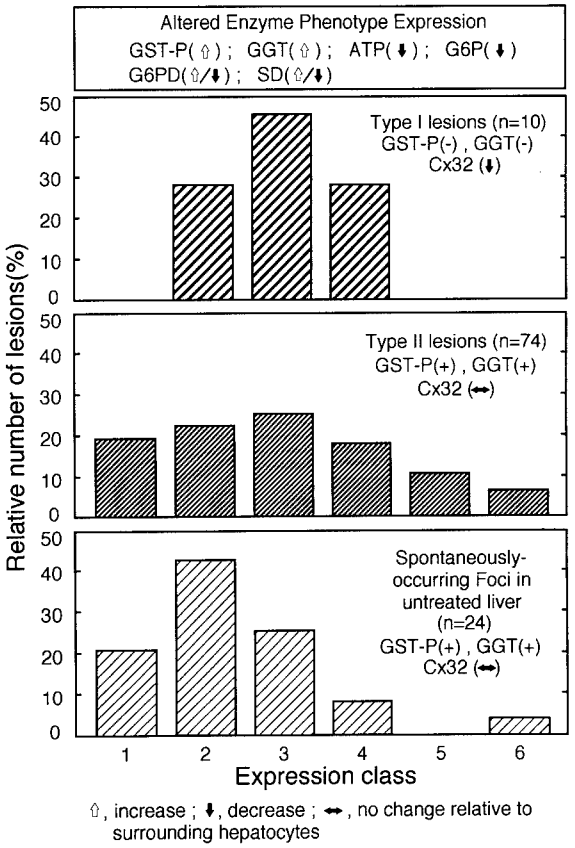


Fig. 3. Altered enzyme expression class distribution of Type I and II lesions in clofibrate-treated liver and spontaneously-occurring foci. GST-P (increased); GGT (increased); ATPase (decreased); G6Pase (decreased); G6PD (increased); SD (increased or decreased); Cx32 (decreased or no change).

alteration of GST-P and/or GGT expression to negative [20]. Type II lesions might include foci initiated by clofibrate but the latter possibility requires further clarification because the question of whether peroxisome proliferators including clofibrate can exert initiating activity has not been clearly elucidated as yet [21,22].

Our earlier findings indicated a Cx32 reciprocity with the increase of cells in S-phase in the regenerating liver, suggesting that proliferating cells lose their capacity to express Cx32, possibly providing suitable conditions for cell division by blocking normal homeostatic control [23]. It has also earlier been reported that Cx32 is decreased in terms of both morphology and function in nitrosamine-induced GST-P positive preneoplastic lesions in the rat [24,25]. Furthermore, in EHEN-induced lesions, the decrease in Cx32 count was shown to significantly correlate with an increase in degree of enzyme expression class, exhibiting an inverse relation to their stage of progression. This result stimulated our interest in whether such a decrease in Cx32 expression might also occur in lesions induced by the non-genotoxic carcinogen, clofibrate, which is known to influence enzyme phenotype expression [18–20]. The results of the present study revealed that the Cx32 spot count in Type I lesions was clearly decreased, irrespective of the altered enzyme expression status. In other words, expression of Cx32 in foci is not dependent on the inducing agent, but rather by some regulatory factor more relevant to neoplastic development [8,10].

In contrast to the Type I lesions, Type II lesions found in the same livers did not show any

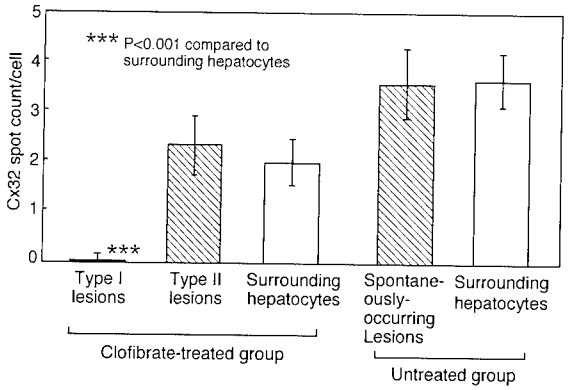
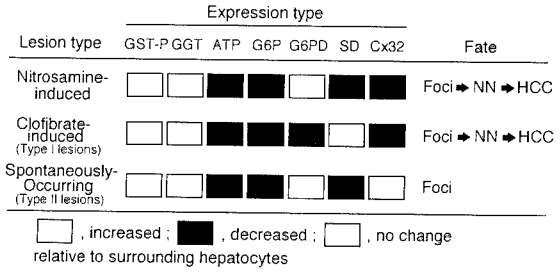


Fig. 4. Numbers of Cx32 spots for hepatocytes of clofibrate-treated and untreated groups. Values for Type II lesions are significantly decreased compared to the surrounding hepatocytes. In contrast, those for Type II and spontaneously-occurring foci do not show a significant difference compared to the respective surrounding hepatocytes.



Phenotype Expression Characteristics of 3 Different Types of Neoplastic Lesions in the Rat Liver

Fig. 5. Schematic presentation of the phenotypes of the three different lesions. It should be noted that irrespective of the enzyme phenotype expressed, lesions exhibiting a decrease in Cx32 expression have the potential to progress to NN and carcinomas.

significant decrease in Cx32 count as compared to the surrounding hepatocytes. Similar observations were made for spontaneously-occurring foci in untreated liver. Furthermore, the altered enzyme expression class profile was essentially the same for both of these groups of lesions, suggesting that Type II lesions may be spontaneous in origin. This is also in line with the quantitative data for numbers of foci per cm^2. Earlier reports indicated that the lack of GJIC between neoplastic cells and surrounding normal cells is essential for the maintenance of the transformed phenotypes [9–12], indicating that an unchanged condition of Cx32 may correlate with potential reversibility of the lesion.

From our previous work and the current study, three types of preneoplastic lesions can be distinguished; (1) nitrosamine-induced lesions, as earlier reported, exhibiting various altered enzyme expression with decreased Cx32 count; (2) clofibrate-induced lesions lacking GST-P and/or GGT with decreased Cx32; (3) spontaneously-occurring lesions exhibiting similar enzyme alterations as their nitrosamine-induced counterparts but no alteration in Cx32 count. It should be noted that lesions exhibiting a decrease in Cx32 expression may have a greater potential to progress to NN and carcinomas (Fig. 5).

Accordingly, the present observations indicate an important role for GJIC in progression in hepatocarcinogenesis, Cx32 expression apparently correlating with cellular independence and growth advantage. In conclusion, the decrease in Cx32 counts may be directly relevant to neoplastic development, irrespective of enzyme phenotype expression of focal lesions and the carcinogens used for their induction.

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Role of blocked gap junctional intercellular communication in non-genotoxic carcinogenesis

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Abstract

Gap junctional intercellular communication mediates the transfer of small molecules from the cytoplasm of one cell to that of neighbouring cells. Connexins are the proteins that form the channels responsible for this type of communication. Aberrant expression and function of connexins are often found in cells exposed to tumor-promoting agents and during carcinogenesis, both in cell culture systems and in tissues freshly removed directly from patients and exposed animals. Transfection of connexin genes into tumorigenic cells often exerts negative growth control, suggesting that connexins act as a family of tumor-suppressor genes. Connexin gene mutations appear to be the cause of two human diseases, i.e. X-linked Charcot-Marie-Tooth syndrome and visceroatrial heterotaxia. Connexin genes are therefore important for the maintenance of homeostasis and thus their dysfunction could lead to various forms of disease.

Keywords: Cell-cell communication; Gap junctions; Connexins; Multistage carcinogenesis; Tumor suppressors

1. Introduction

In tissues, cells communicate directly between each other by exchanging small molecules through gap junctions from cytoplasm to cytoplasm [1]. These small molecules probably include sugars, nucleotides, amino-acids and ions which are important for house-keeping of cells. Second messengers such as calcium, cAMP and inositol triphosphate also pass through gap junctions and participate in the control of tissue homeostasis [2].

The transfer of cytoplasmic molecules between cells is made possible by the juxtaposition of two hemichannels crossing the membranes of two cells in contact. Each hemichannel, called a connexon, is made of six protein molecules, the connexins (Cx). Connexins are members of a multigene family [3]. Their structures are closely homologous, except in their cytoplasmic sequences and especially the length of their carboxy terminal part, where phosphorylation of residues modulates their function, probably by modulating their conformation [4]. Each type of connexin (named according to their molecular weight in kDa) is expressed in particular types of tissue. Although most tissues express several connexin species, the precise combination may play an important role in the maintenance of cell differentiation.

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The function of gap junctions is estimated by direct intracellular microinjection of fluorescent tracers, such as Lucifer yellow, which are able to pass through the transmembrane channels from the microinjected cells into the neighbouring cells in contact. This allows the communication capacity of cells in culture or in tissue slices to be measured [5,6]. Other methods which can be used include metabolic cooperation [7] and electrophysiological means [8].

2. Gap junctional intercellular communication is reduced in tumors and by tumor-promoting agents

Gap junctional intercellular communication (GJIC) is absent or decreased in all cancer cell lines we tested, whatever their origin or the type of connexin expressed. Measurements of GJIC in human cancer cell lines and their normal counterparts in our laboratory are summarized in Table 1. Even if homologous communication among transformed cells is present, heterologous communication between the transformed and non-transformed cells does not occur, as seen in BALB/c 3T3 cells [9]. These data do not seem to be an in vitro artefact since the same was observed in vivo in rat liver tumors and in pieces of human liver tumors removed during surgery [6,10]. The lack of communication is sometimes due to the lack of connexin gene transcription in cancer cells but more often due to post-translational modifications of connexins. In rat liver tumors, for example, there is loss of connexin gene transcription, but not in human liver tumors [10,11]; the connexins are normally expressed but non-functional because they accumulate in the cytoplasm instead of in plasma membranes.

Table 1
Comparison of GJIC (number of communicating cells as tested by dye transfer assay) between human tumor cells and their counterparts

Cell type	Tumor cells	Non-tumoral cells
Mesothelial cells	0–10	18–40
Liver cells	8–10[a]	15–50
Epidermal cells	0–20	30–40

[a] Infected with SV40.

Many types of tumor-promoting agents inhibit the gap junctional communication capacity of various cell types [12]. Molecular studies indicate that defective gap junctional communication in cells exposed to tumor promoters involves aberrant regulation of connexins. For instance, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) causes drastic inhibition of gap junctional communication due to aberrant localization of the connexins in the cytoplasm, as shown by immunocytochemistry. This translocation appears to be the consequence of hyperphosphorylation of residues, which modifies the migration capacity on polyacrylamide gels of the connexin 43 (Cx43) expressed in the relevant cells, as tested by Western analysis [13].

Most studies on the effect of tumor-promoting agents have been confined to in vitro models. We have extended this approach to measurement of GJIC in freshly removed tissue samples. We examined the effect on rat liver intercellular coupling of four agents that are considered to exert liver tumor-promoting activity through different mechanisms: phenobarbital (PB), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs; arochlor 1260), and clofibrate (CF). PB and PCBs are inducers of cytochrome P450s; PCBs induce both PB-type and methylcholanthrene-type P450s [14]. Although CF is a peroxisome proliferation inducer [15], the main biological activities of DDT are not known, except that it has an inhibitory effect on coupling in various types of cells in culture [16]. All four tumor-promoting agents (PB, DDT, PCBs, and CF) exerted inhibition of dye-coupling capacity of rat liver. The strongest effect was observed in the groups treated with PCBs and DDT. PB caused the least inhibition of dye coupling. The most prominent inhibition was found after 2 weeks of exposure in all experimental groups; after 5 weeks of treatment, intercellular coupling slightly recovered in all treated animals [17]. We have also examined the effect of the same agents on the expression of three major liver connexins, Cx26, Cx32 and Cx43, at both mRNA and protein levels. Our results indicate that the decrease in GJIC is associated with aberrant localization of a certain amount of a major liver connexin (Cx32) and

with partial inhibition of expression in hepatocytes of another connexin (Cx26).

The cytoplasmic localization of connexins is not only the consequence of aberrant phosphorylation but may also be due to cell-cell recognition. Mouse epidermal cells communicate through gap junctions when the concentration of extracellular calcium is high. When the calcium level is decreased, the communication capacity drastically decreases. We observed that the induction of communication with increasing calcium concentration is correlated with the mobilization of connexins from the cytoplasm to the plasma membrane. This process is not dependent on calcium by itself but is due to the presence of a cell-cell recognition molecule (E-cadherin) which needs high levels of calcium to be functional. When this protein is not expressed, as in a papilloma cell line we characterized, the cell-cell recognition process is limited and connexins do not move through the cytoplasm. Connexin mobilization is re-induced after the transfection and expression of the E-cadherin gene in this cell line [18].

3. Connexins as tumor-suppressor genes

All the data described above suggest that connexins could act as tumor suppressors. Indeed recently, more direct evidence for the role of GJIC in negative growth control or as a tumor-suppressor element has come from experiments in which connexin genes were transfected into tumorigenic cells. Thus, Cx32 has been shown to retard in vivo, but not in vitro, growth of human hepatoma cells [19]. When Cx43 cDNA was transfected, growth of rat glioma C6 [20] and of chemically transformed mouse 10T1/2 cells [21] was retarded both in vitro and in vivo. In order to examine whether different connexin gene species exert different degrees of tumor-suppressing activity, we characterized the growth characteristics of a gap junction-deficient human cancer cell line, HeLa cells, before and after transfection with cDNA for three different connexins, Cx26, Cx40 and Cx43. All transfected cell lines (three clones transfected with the Cx26 gene, two clones with Cx40, and one with Cx43) showed establishment of GJIC. Two of the Cx26-transfected clones showed significantly slower growth compared with the parental HeLa cells. When transfectants were grown in soft agar, the three Cx26-transfected clones grew much less than the other transfectants or the parent HeLa cells. When injected into nude mice, the two Cx26 clones which exhibited the highest amount of Cx26 transcript induced almost no tumors, whereas the other transfectants, including the Cx26 clone which exhibited the lowest amount of Cx26 transcript, were tumorigenic. Among transfectants with various connexin genes, there was no clear inverse correlation between their GJIC and tumorigenicity. GJIC levels were significantly higher in tumors induced in nude mice by Cx26 transfectants. These results suggest that all of the connexin genes examined could induce recovery of GJIC of HeLa cells, but that only the Cx26 gene exerts strong negative growth control on HeLa cells; thus, this connexin gene may have different functions from other connexin genes. Cervical tissues, from which HeLa cells were originally derived, express Cx26 as a major connexin (Fig. 1), suggesting that the connexin type expressed under normal physiological conditions plays an important role in the suppression of tumorigenicity [22].

4. Connexin gene mutations in tumors

While the inhibition of GJIC in toxicology has always been approached as a non-genotoxic process, it is possible that genotoxic mechanisms, such as mutations of connexin genes, lead to GJIC impairment. We have therefore started to search for Cx32 gene mutations in human and rodent tumors using SSCP. In 20 human liver tumors from France [10] and 22 human gastric tumors from Russia [23], we found no sign of mutations in the coding regions. In all of the same liver tumors, we found reduced GJIC and/or aberrant localization of Cx32 proteins. However, among seven rat liver tumors induced by a nitrosamine, we found one sample with a mutation at codon 220 of the Cx32 gene. This sample also showed aberrant localization of connexin molecules; Cx32 proteins were localized in the nuclear region rather than at cell-cell contact areas. Our subsequent studies indicated that this

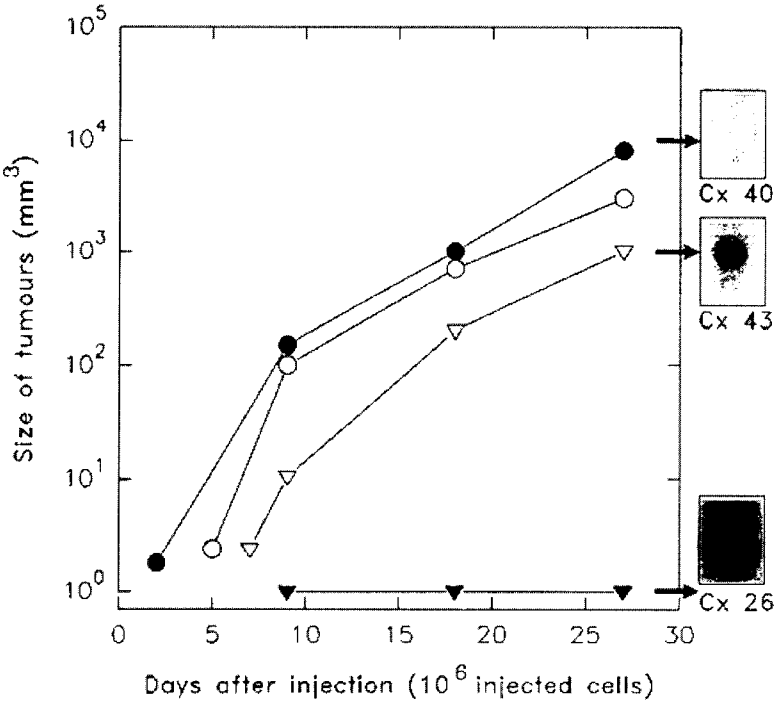


Fig. 1. Differential tumor suppressor effects of different connexin species on HeLa cells. The tumorigenicity of HeLa cells transfected with the Cx26 gene, but not those with Cx40 or Cx43 genes, was suppressed. The tumor suppressive effects were correlated with the level of connexin gene expression in the cervical tissue from which HeLa cells had been originally isolated as shown in the Northern analyses of the right side of the figure; Cx26 is the most abundantly expressed connexin gene in the cervix.

mutation is functionally silent, at least in HeLa cells, after transfection. While these results suggest that GJIC may be more often impaired by non-genotoxic mechanisms, it should be noted that the Cx32 gene is located on the X chromosome, where no other known tumor-suppressor genes have been found. Mutations of the Cx37 gene have been reported in cell lines derived from murine lung tumors [24], and it seems possible that future studies will reveal mutations of other connexin genes in certain types of tumors.

5. Connexin gene mutations and other human diseases

Recent studies have begun to show that the mutations of connexin genes may be responsible for certain human diseases. For example, some types of viscero-atrial-heterotaxia syndrome are caused by germ-line mutations in the Cx43 gene, resulting in loss of response to cAMP-dependent protein kinase [25]. Moreover, germ-line mutations of the Cx32 gene have been reported to be responsible for X-linked Charcot-Marie-Tooth (CMT) disease [26]. As shown in Fig. 2, mutations can occur at various regions of coding sequences.

In order to see whether the Cx32 gene mutations found in CMT patients impair GJIC, we introduced four different Cx32 mutations discovered in patients with CMT disease into wild-type cDNA and transfected them into non-communicating HeLa cells. Cells transfected with the wild-type Cx32 gene, but not those transfected with genes carrying three different base substitution mutations (i.e. Cys 60 to Phe, Val 139 to Met and Arg 215 to Trp), recovered GJIC, as estimated by direct microinjection of Lucifer yellow. Unexpectedly, however, cells transfected with a nonsense mutant at codon 220 also had restored

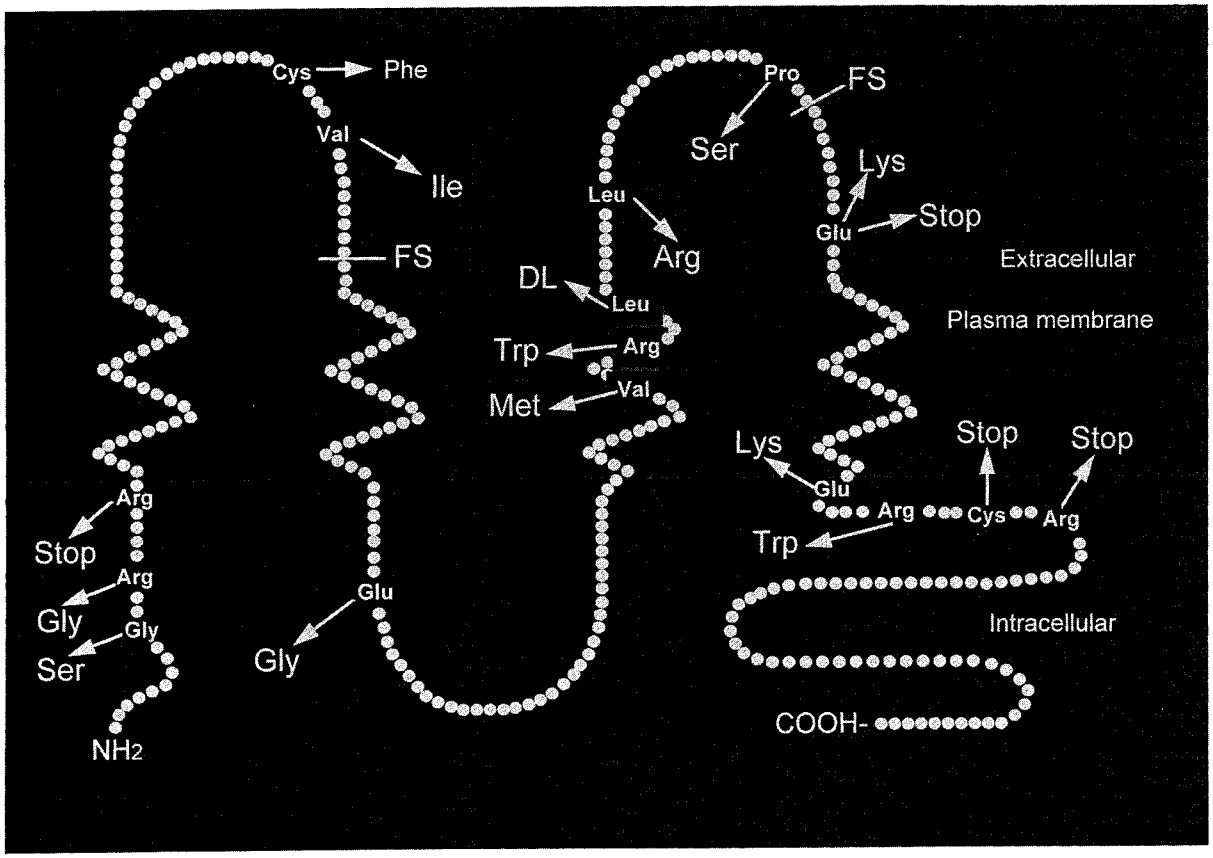


Fig. 2. Connexin 32 mutations found in X-linked Charcot-Marie-Tooth patients. Note that the mutations do not affect any particular region of the protein (FS, frameshift; DL, deletion).

GJIC. These results suggest that most, if not all, Cx32 gene mutations associated with CMT disease do indeed impair GJIC, which may be the cause of this disease.

The fact that connexin gene mutations lead to different human diseases strongly suggests that GJIC is responsible for a range of biological functions. This may not be surprising; since GJIC appears to play a central role in maintenance of homeostasis, its impairment is likely to result in various physiological disorders. This is also reflected in recent results showing that Cx43 or Cx26-null mice die prematurely [27]. From the toxicological point of view, therefore, GJIC may be a good marker to assess risks of various agents.

Acknowledgements

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Toxicology Letters

Recent developments in methanol toxicity

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Abstract

The disposition of methanol and its putative toxic metabolite formate has been studied in humans, non-human primates, and rodents after exposure to high, neurotoxic doses. The rate at which rodents detoxify formate is more rapid than that of primates. Formate, an endogenous biological substrate, is detoxified by metabolism to CO_2 via a tetrahydrofolate-(THF) dependent pathway. Species with high hepatic THF levels, such as rodents, are less sensitive to the neurotoxic effects of large methanol doses compared with species with low THF levels, such as primates. Data on the capacity of primates to detoxify formate derived from inhalation of low levels of methanol are critical for assessing human risk from methanol fuels. Female cynomolgus monkeys exposed to low concentrations of [^{14}C]methanol (10–200 ppm) for 2 h have blood levels of methanol-derived formate that are 100- to 1000-fold lower than endogenous levels of formate. Healthy human volunteers exposed at rest or during exercise to 200 ppm methanol for 6 h or exposed to 20 mg/kg orally have elevated blood levels of methanol, but blood formate concentrations are not significantly increased above endogenous concentrations. Deficiencies in THF may prolong blood levels of formate and increase the likelihood of toxic effects. Limited studies in non-human primates with low THF levels exposed to 900 ppm methanol for 2 h have shown that concentrations of methanol-derived formate in blood remain below endogenous levels. Thus human populations may not be at added risk of neurotoxic effects resulting from exposure to low levels of methanol.

Keywords: Methanol; Formate; Folate; Non-human primates

1. Introduction

Methanol (wood alcohol) has the potential to become a major automotive fuel in the United States in the next century [1]. Emissions of methanol can arise from its release as uncombusted fuel in automobile exhaust or from its evaporation during refueling and after the engine is stopped. The United States Environmental Protection Agency has modeled methanol exposure levels that might occur under specific conditions of use [1]. For example, if 100% of all automobiles were powered by methanol-based fuels, simulation models predict concentrations of methanol in urban streets, expressways, railroad tunnels or parking garages ranging from a low of 1 mg/m^3 (0.77 ppm) to a high of 60 mg/m^3 (46 ppm). Predicted methanol concentrations in a personal garage range from 2.9 mg/m^3 to 50 mg/m^3 while predicted concentrations during refueling of vehicles range from 30 to 50 mg/m^3. For reference purposes, the threshold limit value (TLV) of the American

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Conference of Governmental Industrial Hygienists (ACGIH) for worker exposure to methanol over an 8-h work day is 260 mg/m^3 (200 ppm).

A large body of evidence on the acute toxicity of methanol has been accumulating since the early 1900s, when exposure of humans to relatively large acute doses of methanol occurred either through accidental or intentional ingestion, percutaneous absorption or inhalation. In the early part of the century, painters used methanol as a cleaning fluid or as a dilution agent for shellac, varnish and paint. Methanol was also used as a solvent by hatters, dyers, shoemakers, and brass finishers and in the manufacture of rubber tires [2]. During this time, about 100 cases of vision impairment or death from inhalation of methanol vapors were reported. These cases demonstrate that acute human exposure to methanol can result in blindness, metabolic acidosis, and death.

Formate is the metabolic product responsible for the acute toxic effects of methanol [3–5]. Formate is detoxified by a multistep pathway to carbon dioxide (CO_2) [6]. In all species studied, this is achieved through a tetrahydrofolate-(THF) dependent pathway. Folate is an essential vitamin found in fresh fruits and vegetables and is the building block of THF. Rodents have higher concentrations of liver THF compared with primates and therefore are more efficient in the metabolism of formate to CO_2 than humans and non-human primates. The faster rate of formate removal in rodents prevents accumulation of formate above endogenous levels at any methanol dose. Therefore rodents are not susceptible to methanol-induced metabolic acidosis or ocular toxicity from exposures that cause these effects in humans and non-human primates.

2. Low level methanol exposure

The issue of blood methanol and formate concentrations following inhalation exposure to methanol vapors has been addressed by Horton et al. [7], who exposed male rhesus monkeys to concentrations of methanol ranging from 200 ppm to 2000 ppm for 6 h. Although these levels are higher than those expected during normal fuel use they still enable us to get a perspective on the issue. Concentrations of methanol and formate in the blood of the primates were measured for up to 18 h after the end of the 6-h inhalation exposure. The highest blood methanol concentrations occurred immediately following the 6-h inhalation exposures and declined steadily thereafter. The concentrations of formate in the blood of the monkeys exposed to these methanol vapors were not elevated above the endogenous blood formate concentrations determined at the start of the exposure (Fig. 1). Blood concentrations of formate varied considerably among the individual monkeys and at various times up to 18 h after the end of the exposure. However, these formate concentrations were not influenced by either time or methanol exposure concentration, suggesting that the formate levels, although variable, were not elevated above endogenous levels by exposure to inhaled methanol. The highest methanol exposure concentration used by Horton et al. [7], 2000 ppm, is 10 times higher than the current time weighted average (TWA)-TLV for methanol of 200 ppm.

Although these studies showed that blood formate concentrations were not increased above endogenous levels following exposure to 200 ppm methanol, the contribution of methanol-derived formate to the total body pool of formate could not be determined. This issue was addressed by Dorman et al. [8] using female cynomolgus monkeys which were exposed to a

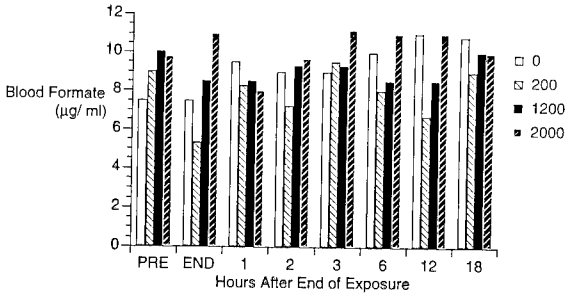


Fig. 1. Blood concentrations of formate in male rhesus monkeys prior to (pre) and after (end) exposure to 0 (control), 200, 1200, or 2000 ppm methanol for 6 h. Data represent average formate concentrations for three monkeys taken from Horton et al. [7].

range of inhalation exposure concentrations of methanol spanning both workplace and environmental exposures. Carbon-14 radiolabeled methanol was incorporated into the exposure atmosphere to aid in measuring concentrations of methanol and formate in blood and excretory products both during and after exposure to the inhaled methanol. The results summarized in Table 1 indicated that a 2-h exposure to [^{14}C]methanol concentrations up to 900 ppm produced a maximum blood [^{14}C]formate concentration that was only a fraction of the endogenous formate level (100–200 nmol/ml) and several orders of magnitude lower than levels of formate known to be toxic (>7000 nmol/ml). These studies suggest that the maximum blood concentration of formate resulting from exposure to low concentrations of methanol, such as those that might result from the use of methanol as an alternative fuel, is insignificant when compared with endogenous formate levels.

Recent experimental studies in humans exposed to concentrations of methanol at the TWA-TLV are consistent with results of experiments in non-human primates. For example, Lee et al. [9] exposed healthy volunteers to methanol concentrations of 200 ppm for 6 h. The subjects were either at rest or engaged in mild exercise. These investigators were able to observe a 3.5- to 4-fold increase in the peak methanol concentration in blood of the exposed subjects compared to their preexposure levels, indicating that they had absorbed some of the methanol vapor. However, when these investigators analyzed the concentrations of formate in the blood of these individuals, they were not able to detect a change in the blood formate concentration compared to the preexposure value. Similar results were observed in humans exposed to methanol while resting or exercising. Most recently, d'Alessandro et al. [10] measured formate levels in urine and serum after controlled methanol exposures of healthy volunteers at the threshold limit value (200 ppm). These investigators also could not detect any increase in formate in urine or serum due to methanol exposure compared to the control values. Taken together, results of studies in humans and non-human primates exposed to concentrations of methanol ranging from 10 to 2000 ppm suggest that exposure to methanol vapors during the normal use of methanol fuel does not pose an unacceptable risk to healthy adults.

3. Sensitive subpopulations

As noted previously, the hepatic stores of folate in the liver are an important determinant for predicting whether or not a species is sensitive to methanol-induced acute toxicity [6,11,12]. Studies were conducted to determine how methanol is metabolized by non-human primates with reduced stores of folate [8]. The same female cynomolgus monkeys that were used in the previously described studies of Dorman et al. [8] were placed on a folate-devoid diet until reduced folate levels in red blood cells of these monkeys were observed. The monkeys were then exposed to 900 ppm of [^{14}C]methanol for 2 h. Even with a reduced folate status, monkeys exposed to 900 ppm of [^{14}C]methanol for 2 h still had peak concentrations of [^{14}C]methanol-derived formate that were well below the endogenous formate levels and orders of magnitude lower than levels that produce acute methanol toxicity (Table 1). Although these results only

Table 1
Measured peak methanol and formate concentrations in primates exposed to [^{14}C]methanol[a]

[^{14}C]Methanol exposure (ppm)	Blood [^{14}C]methanol (μM)	Blood [^{14}C]formate (μM)
10	0.65 ± 0.3	0.07 ± 0.02
45	3.0 ± 0.8	0.25 ± 0.09
200	21 ± 16	2.3 ± 2.9
900	106 ± 84	2.8 ± 1.7
900 (folate-deficient)	211 ± 71	9.5 ± 4.7

[a] Data taken from Dorman et al. [8].

represent a single exposure and therefore preclude broad generalizations, they do suggest that the body contains sufficient folate stores to effectively detoxify small doses of methanol-derived formate from inhalation of methanol resulting from its normal use as an automotive fuel.

4. Developmental toxicity

Recent studies conducted in rodents [13–15] have demonstrated that methanol exposure impairs neural tube closure. The concentrations of methanol used in the teratogenicity studies greatly exceed estimates of likely exposure scenarios to methanol vapors relating to its use as an automotive fuel. Nonetheless, given what is known about the dramatic differences in the way rodents and primates metabolize methanol and formate, is the biochemical basis for the observed teratogenic effects in rodents relevant for humans?

Formate seems to play no apparent role in the development of methanol-induced exencephaly in mice [16]. Mice exposed to 15 000 ppm methanol for 6 h developed exencephaly and had high concentrations of methanol (223 ± 23 mM) in maternal plasma but did not accumulate formate in either plasma or conceptuses. Additionally, animals given a large single oral dose of sodium formate (750 mg/kg) did not develop exencephaly or decidual swelling in excess of 2.5 mm.

The relationship between the folate status and developmental neurotoxicity is well recognized. Sakanashi and coworkers (17) reported an increase in methanol-induced exencephaly and other terata in CD-1 mice on a folate-deficient diet and also reported that folate supplementation ameliorated those adverse developmental effects. Research on the relationship between methanol exposure and fetotoxicity should help answer further questions about the risk of exposure to low concentrations of methanol if it is used as an alternative fuel.

Acknowledgements

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Toxicology Letters

Toxicokinetics and acute effects of MTBE and ETBE in male volunteers

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Abstract

Methyl tertiary butyl ether (MTBE) is widely used in gasoline as an oxygenator and octane enhancer. There is also an interest in using the ethyl tertiary butyl (ETBE) and methyl tertiary amyl (TAME) ethers. We measured the blood, water, and olive oil/air partition coefficients in vitro of MTBE, ETBE, TAME and tertiary butyl alcohol (TBA), a metabolite of MTBE and ETBE. The results indicate similar uptake and distribution behavior for the three ethers and a slight affinity for fatty tissues. The partition coefficients of TBA indicate that this metabolite is not excreted via the lungs to any great extent and that it is preferentially distributed in body water. Further, we exposed 10 healthy male volunteers to MTBE vapor at 5, 25 and 50 ppm for 2 h during light physical exercise. Uptake and disposition were studied by measuring MTBE and TBA in inhaled and exhaled air, blood and urine. Low uptake, high post-exposure exhalation, and low blood clearance indicate slow metabolism of MTBE relative to many other solvents. A low recovery of TBA in urine (below 1% of uptake) indicates further metabolism of TBA. The concentration of MTBE and TBA in blood was proportional to exposure level suggesting linear kinetics up to 50 ppm. The half life of 7–10 h in blood and urine indicates that TBA would be more suitable than the parent compound as a biomarker for MTBE exposure. Subjective ratings (discomfort, irritative symptoms, CNS effects) and eye (redness, tear film break-up time, conjunctival damage, blinking frequency) and nose (peak expiratory flow, acoustic rhinometry, inflammatory markers in nasal lavage) measurements indicated no or minimal effects of MTBE.

Keywords: Human chamber exposure; Methyl tertiary butyl ether; Ethyl tertiary butyl ether; Methyl tertiary amyl ether; Liquid/air partition coefficients; Tertiary butyl alcohol

1. Introduction

Methyl tertiary butyl ether (MTBE) is widely used in gasoline as an oxygenator and octane enhancer. There is also an interest in using the ethyl tertiary butyl (ETBE) and methyl tertiary amyl (TAME) ethers [1]. Experiences of nausea, headache, and irritation, in some regions have allegedly been associated with the use of and exposure to gasoline containing MTBE [2,3].

MTBE, and probably also ETBE, is metabolized to tertiary butyl alcohol (TBA) in rat liver microsomes [4] and by rat lung tissue and blood [5]. In rats, TBA is further metabolized to 2-

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methyl-1,2-propanediol and α-hydroxyisobutyric acid, and these metabolites are subsequently excreted in urine [5]. TBA has also been demonstrated in blood samples from humans exposed to MTBE [2,6].

The alleged acute effects associated with exposure to MTBE together with the search for suitable biomarkers of exposure to gasoline prompted us to initiate a research project on the toxicokinetics and acute effects of ether additives in humans. The project consists of the following parts: (1) Determine partition coefficients in vitro, for use in physiologically-based pharmacokinetic (pbpk) modeling; (2) Study toxicokinetics and acute effects in experimental chamber exposure; (3) Develop a pbpk model, as a basis for biomonitoring and target dose estimates.

This paper summarizes the methods and preliminary results with respect to partition coefficients of MTBE, ETBE, TAME, and TBA and toxicokinetics and acute effects of MTBE in humans. The results will be presented in detail elsewhere ([7] and Nihlen et al. (in preparation), respectively). The human chamber exposure study with MTBE is currently being repeated for ETBE.

2. Materials and methods

2.1. Subjects

Blood for determination of partition coefficients was sampled from five female and five male caucasians. Ten male caucasian volunteers (age 23–51 years, body weight 70–90 kg) participated in the chamber exposure study. The subjects were healthy as judged by medical examination and standard clinical blood chemistry. All were nonsmokers and were not taking any drugs at the time of the experiment. They had no history of excess usage of alcohol or other drugs, liver disease, or occupational exposure to MTBE or other organic solvents. Further, the volunteers were not using contact lenses, and had no history of eye diseases or allergy. They had to refrain from alcoholic beverages starting 48 h prior to and throughout the experiment. The studies were approved by the regional ethical committee at the Karolinska Institute, Solna, and were only performed after informed, written consent.

2.2. Partition coefficients

Liquid/air partition coefficients were determined by the closed vial technique as described elsewhere [7]. In brief, 0.5 ml olive oil or 2 ml pooled human blood or physiological saline was added to a 22-ml gas tight glass vial. After addition of 2 μl of a water mixture of the three ethers the vial was allowed to equilibrate at 37°C for 40 min. The gas phase was analyzed for ethers by head-space gas chromatography using a Poraplot Q capillary column and flame ionization detection. Partition coefficients were obtained by comparing peak areas between the sample vial and a reference vial without liquid phase.

2.3. Exposure conditions

Two subjects were exposed at a time in a 12-m^3 exposure chamber with 18–20 air exchanges per hour. Exposures were conducted for 2 h during light physical exercise (50 W) on a bicycle ergometer. Each subject was exposed on three different occasions, at the nominal levels 50 ppm (the current Swedish permissible exposure level [8]), 25 ppm, and 5 ppm. Actual levels were continuously monitored by Fourier transform infrared spectrophotometry. Actual levels were 49 ± 0.8 (mean $\pm$S.D.), 24 ± 2.6, and 4.8 ± 1.8 ppm.

2.4. Sampling and chemical analyses

Mixed exhaled air was sampled before, during, and up to 24 h (48 h at 50 ppm) after exposure. Expired breath was conducted via a mouth piece fitted to a one-way valve into a mixing chamber and subsequently absorbed on an Anasorb 747 charcoal tube. The tube was desorbed with carbon disulfide and analysed by gas chromatography with flame ionization detection using a CP Sil 8, bonded phase, capillary column.

Capillary blood was sampled before, during,

and up to 24 h (48 h at 50 ppm) after exposure. The blood samples were analysed for MTBE and TBA by head-space gas chromatography (Poraplot Q capillary column, flame ionization detection). Standards were prepared using venous blood sampled immediately prior to exposure.

Before exposure and up to 24 h (48 h at 50 ppm) following exposure, the subject was asked to empty the bladder completely. After measuring the urine volume the urine samples were analyzed in the same way as the blood samples. Standards were prepared using urine voided before exposure.

2.5. Symptoms questionnaire

At certain time intervals the subject was asked to answer a short questionnaire with 10 questions related to symptoms of central nervous system effects (headache, fatigue, feeling of sickness, dizziness and intoxication), smell, difficulty to breath, and irritation of the eyes, nose and throat. Answers were given on a 100-mm visual analog scale graded from 'not at all' to 'almost unbearable' [10]. Scorings were made on eight occasions, prior to, during, and up to 4 h after exposure.

2.6. Eye effects

Blinking frequency was monitored by counting the number of blinkings in 3 min on video recordings made before, five times during exposure, and twice after exposure.

Eye redness was scored according to Kjaergaard [10] by assessing the degree of injection in the bulbar conjunctiva. Scoring was made on diapositives so that the examinator was unaware of the exposure conditions. Eye photographs were taken on the day prior to exposure (day 0), before and after exposure on day 1 and on days 2 and 3.

Tear film stability was assessed by measuring the tear-film break up time after instilling fluorescein into the lower conjunctival sac [11]. Tear film stability was also estimated by recording the self reported tear-film break-up time, i.e. the time from the last blinking until the subject recognizes the sensation of a dry spot in the eye [12]. Break-up times were measured once on day 0, before, during and after exposure on day 1 and on days 2 and 3.

Epithelial damage of the cornea and conjunctiva was assessed by staining dead and degenerated cells of the ocular surface with Lissamine green [11,13]. Vital staining was carried out once daily, at days 0, 1 (4 h after exposure), and 2.

2.7. Nasal effects

Nasal and mouth peak expiratory flow (PEF) rates were compared to obtain the blocking index, a measure of nasal airway resistance [14]. The blocking index (BI) was calculated as:

$$BI = (\text{mouth PEF} - \text{nasal PEF})/\text{mouth PEF}$$

Nasal swelling was also assessed by estimating nasal volume and smallest nasal cross-sectional area by acoustic rhinometry [15]. Nasal swelling was investigated before, directly after, and up to 24 h after exposure.

Markers of nasal inflammation were studied in nasal lavage (5 ml room tempered, sterile, physiological saline). The following parameters were studied in the nasal lavage: cell count (leukocytes, erythrocytes, epithelial cells), eosinophilic cationic protein, myeloperoxidase, lysozyme, and albumin. The values were calculated both with and without volume correction for differences in fluid recovery in the lavage. Nasal lavages were made on days 0, 1 (before and after exposures), 2, and 3.

Questionnaires and PEF measurements were carried out at all exposure levels and acoustic rhinometry at 25 and 5 ppm. The other effect parameters were only studied at 50 ppm.

2.8. Statistical evaluation

Individual blood levels of MTBE were fitted to the analytical expression of linear pharmacokinetic four-compartment model, with zero order order input. Optimization was made by minimizing the unweighted sum of squares using Microsoft Excel 4.0 and the Solver add-in macro

on a Macintosh computer. The decay curves of MTBE in urine and TBA in blood and urine were fitted to mono- and biexponential functions, respectively, again with the use of Solver.

Indicators of acute effects were analyzed by the repeated measurements analysis of variance (ANOVA) with the use of Superanova 1.11 on Macintosh. A P-value of 0.05 was choosen as the level of significance.

3. Results and discussion

3.1. Partition coefficients

The measured partition coefficients are given in Table 1. The values are comparable to those of other solvents, such as diethyl ether and toluene. The blood/air partition coefficients of between 12 and 18 indicate efficient uptake from inhaled air as well as excretion via exhaled air. The somewhat higher oil/air partition coefficients of 120–340 suggest a 7- to 20-fold accumulation in fatty tissues compared to blood. In contrast, the very high blood/air partition coefficient of TBA of 460 suggests that this metabolite is not excreted via the lungs to any appreciable extent. The low oil/blood coefficient of 0.36 suggests that TBA is preferentially distributed in body water.

3.2. Toxicokinetics

During exposure to MTBE vapor at 5 ppm the concentration of MTBE in blood increased rapidly and appeared to reach a plateau value of approximately 1.3 μmol/l towards the end of the 2-h exposure (Fig. 1). During exposure to 25 and 50 ppm, blood MTBE levelled off but plateaus were not reached. The blood levels at the end of these exposure levels were approximately 6.5 and 13 μmol/l, respectively. Toxicokinetic parameters for MTBE are given in Table 2. The apparent variability in relative respiratory uptake (32–42%) and the amount exhaled post-exposure (20–33% of inhaled amount) may be due to the limited time intervals of about 5 min each during which exhaled air samples were collected. Post-exposure decay in blood could be separated into three phases with half lives of approximately 10 min, 1.5 h, and 19 h. In urine, two half lives of 20 min and 3 h were identified. As little as 0.1% of the inhaled amount was excreted unchanged in urine in 24 h. The toxicokinetic calculations indicate that the clearance of exhalation is nearly as high as clearance of metabolism.

In contrast to MTBE, blood TBA continued to increase during the 2-h MTBE exposure, then levelled off and started to decline about 6 h later (Fig. 2). The post-exposure half life in blood (10 h) was similar to that in urine (7–9 h) (Table 3). The slower elimination of TBA suggests that this metabolite is more suitable than MTBE itself as a biological exposure marker. Less than 1% of the absorbed dose of MTBE was excreted as

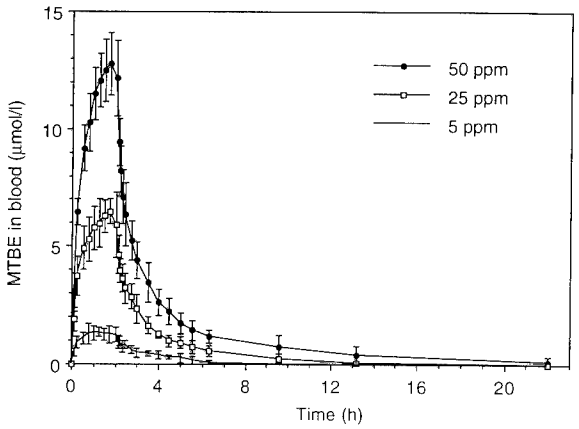


Fig. 1. Methyl tertiary butyl ether in blood sampled from 10 male volunteers during and after expoure to 5, 25, and 50 ppm MTBE for 2 h. Vertical lines indicate ±one standard deviation.

Table 1
Liquid/air partition coefficients determined in vitro at 37°C (summarized from [7])

	$\lambda_{blood/air}$ [a]	$\lambda_{water/air}$	$\lambda_{oil/air}$	$\lambda_{oil/blood}$ [b]
MTBE	17.7	15.2	120	6.8
ETBE	11.7	8.3	190	16.2
TAME	17.9	11.9	337	18.8
TBA	462	603	167	0.36

[a] Pooled blood from five men and five women.
[b] Calculated as $\lambda_{oil/blood} = \lambda_{oil/air}/\lambda_{blood/air}$.

Table 2
Toxicokinetic descriptors of MTBE ($n = 10$)

	Nominal exposure level (ppm)		
	5	25	50
Relative uptake (%)	42	32	41
Exhaled post-exposure (%)	29	33	20
Exhalatory clearance (l/min)	0.30	0.42	0.31
Metabolic clearance (l/min)	0.47	0.43	0.65
$t_{1/2}$ in blood, 1st phase (min)	12.2	9.6	8.6
2nd phase (h)	1.5	1.6	1.4
3rd phase (h)	21	20	17
Excreted in urine in 24 h (% of inhaled)	0.11	0.09	0.11
$t_{1/2}$ in urine, 1st phase (min)	16	21	22
2nd phase (h)	3.1	3.0	3.1

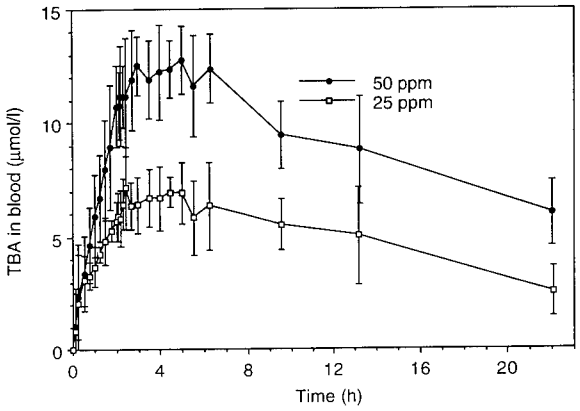


Fig. 2. TBA in blood sampled from 10 male volunteers during and after exposure to 25 and 50 ppm MTBE for 2 h. Vertical lines indicate ±one standard deviation.

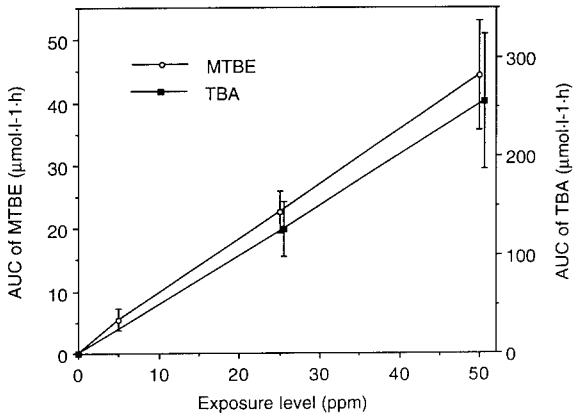


Fig. 3. Area under the concentration-time curve (AUC) of MTBE and TBA in blood versus exposure concentration of MTBE. Vertical lines indicate ±one standard deviation.

Table 3
Toxicokinetic descriptors of TBA upon exposure to MTBE (mean ± S.D., $n = 10$)

	Nominal exposure level (ppm)		
	5	25	50
$t_{1/2}$ in blood (h)	–	10.1	10.2
$t_{1/2}$ in urine (h)	–	8.9	7.4
Excreted in 24 h (% of inhaled)	0.51	0.77	0.55

TBA in urine within 24 h. This low percentage may indicate further metabolism of TBA, as previously shown in rats [5].

The area under the concentration time curves (AUC) of MTBE as well as TBA in blood were linearly related to the MTBE exposure level (Fig. 3), suggesting that the toxicokinetics are linear up to at least 50 ppm.

3.3. Acute effects

With the exception of the rating of solvent smell and nasal blocking index, none of the measurements of acute effects differed significantly from control levels and no effect over time or with increasing exposure level was seen. The subjective rating of solvent smell increased dramatically as the volunteers entered the chamber, and then declined slowly with time during exposure. In addition, the rating of sol-

vent smell increased with increasing exposure level. These effects were highly significant in repeated measures ANOVA (P-values of 0.0001 and 0.001, respectively), illustrating the potential of the questionnaire to pick up symptoms. Blocking index, an indicator of nasal swelling, increased over time during exposure ($P = 0.03$), but no exposure-response relationship was seen, indicating that MTBE was not the cause. The observed increase in blocking index over time may be caused by factors related to the exposure, such as the bicycle exercise or diurnal variations. The nasal swelling observed by PEF measurements could not be confirmed by acoustic rhinometry at 25 and 5 ppm.

Acknowledgements

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Toxicology Letters 82/83 (1995) 719–724

Toxicology Letters

Health effects of inhaled tertiary amyl methyl ether and ethyl tertiary butyl ether

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Abstract

Government specifications on the oxygen content of motor gasoline sold in certain areas of the USA have resulted in commercialization of the oxygenate fuel additives tertiary amyl methyl ether (TAME) and ethyl tertiary butyl ether (ETBE). TAME and ETBE were evaluated in 4-week rat inhalation studies sponsored by Amoco Corporation. Target vapor concentrations were 0, 500, 2000, or 4000 ppm for 6 h per day, 5 days per week, for 4 weeks. TAME exposure at 4000 ppm resulted in 25% mortality, apparently as a consequence of severe CNS depression. Body weight gain was decreased in the TAME high dose male rats. In contrast, no ETBE exposed animals died during the study and no changes in body weight gain were observed. Significant effects on functional observational battery (FOB) parameters were only found in the TAME high and mid-dose groups immediately after exposure. All affected FOB parameters were normal by the next day. Both TAME and ETBE exposures significantly increased relative liver weights in the high and mid-dose groups. However, no treatment-related histopathologic findings were noted for either compound. Clinical chemistry and hematology findings were absent with ETBE exposure and minimal with TAME exposure. The results indicate that 500 ppm was a NOAEL for both compounds in these studies.

Keywords: ETBE; TAME; MTBE; Oxygenate

1. Introduction

The 1990 Amendments to the USA Clean Air Act (CAA) required increased fuel oxygen content in gasoline sold in certain areas of the country. Starting in 1992, areas that failed to meet the National Ambient Air Quality Standard (NAAQS) for carbon monoxide had to use gasoline containing a minimum of 2.7 wt.% oxygen during the winter. This oxygen requirement was the only change in the gasoline specifications. This type of gasoline has been referred to as 'wintertime oxygenated gasoline.' Starting in 1995, nine metropolitan areas that failed to meet the NAAQS for ozone had to use gasoline containing 2.0% oxygen year round. However, the oxygen content was not the only gasoline specification change required. In addition, the benzene concentration had to be below 1.0 wt.%, the maximum allowable fuel vapor pressure was

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reduced, and the gasoline had to provide 15–25% reductions in 'air toxics' emissions. This type of gasoline is referred to as Federal 'reformulated gasoline' or RFG. Gasoline that is sold in areas where these fuel specifications do not apply is referred to as 'conventional' gasoline.

The chemicals blended with gasoline hydrocarbons to meet the required oxygen content of the fuel are referred to as 'oxygenates.' In the USA, the oxygenates used most often are methyl tertiary butyl ether (MTBE) and ethanol. They currently comprise over 90% of the market. Two other oxygenates whose use may increase are tertiary amyl methyl ether (TAME) and ethyl tertiary butyl ether (ETBE). The chemical structures of TAME and ETBE are shown in Fig. 1.

TAME is manufactured from isoamylene and methanol feedstocks. The principle source of isoamylene is the C5-olefin stream from a crude oil refining process called fluid catalytic cracking. The C5 stream from this process would normally go into gasoline. TAME manufacturing provides a refinery with a way to reduce the gasoline vapor pressure, reduce the light olefin content of gasoline, and create a high octane gasoline blending component (Table 1). However similar benefits could also be obtained by using the C5-olefins in the refinery's hydrocarbon alkylation process. The decision to build TAME manufacturing capacity is significantly influenced by the regulatory mandate requiring oxygenates in gasoline.

ETBE is the ethyl analog of MTBE. In many cases, current manufacturers of MTBE could produce ETBE by changing the alcohol feedstock and re-optimizing the production process. The low vapor pressure and high octane of ETBE make it a good gasoline blending component (Table 1). However, the high cost of ethanol compared to methanol limits the amount of ETBE produced commercially. Whether significant amounts of ETBE are ever produced will depend on regulatory decisions including tax subsidies and possible mandatory use of 'renewable' oxygenates.

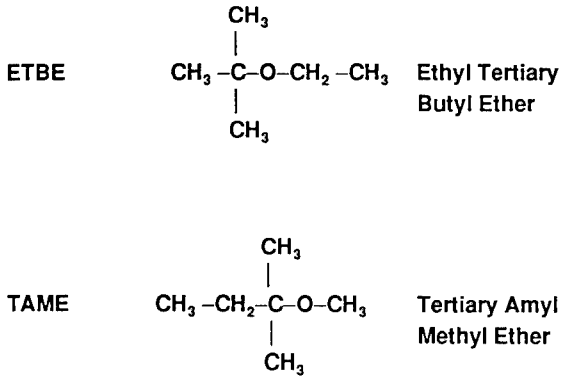


Fig. 1. Chemical stuctures of TAME and ETBE.

2. General methods

The 4-week inhalation toxicity studies on TAME [1] and ETBE [2] described in this paper were sponsored by Amoco Corporation and performed by IIT Research Institute. Abstracts of this work have been presented previously [3,4].

Both studies on TAME and ETBE used Sprague–Dawley rats approximately 9–11 weeks of age. Ten animals per sex per dose were used for ETBE and 14 per sex per dose for TAME. For both materials the test duration was 6 h per day, 5 days a week, for 4 weeks. The exposures were conducted in 1 cubic meter glass and

Table 1
Comparison of physical properties of TAME, ETBE and MTBE

	Boiling point, °F	Blending vapor pressure, psi	Blending octane No.	Water solubility (%)	Estimated Henry's Law constant
TAME	187	2	105	1.2	23
ETBE	163	4	111	1.2	50
MTBE	131	8	110	4.3	30

stainless steel chambers and the test material concentrations within each chamber were measured by infrared (IR) analysis. Target exposure concentrations were 500, 2000, and 4000 ppm. All rats were observed at least once daily, 7 days a week. Body weights were measured at the initiation of the study, weekly during the exposure, and immediately before termination of the animal. For all quantitative parameters, the data were analyzed using both multivariate and univariate two-factor fixed-effects analyses of variance. Quantal data for functional observational battery (FOB) parameters were analyzed using chi-square. A minimum significance level of $P < 0.05$ was used in all comparisons.

3. Neurotoxicity assessment

In addition to daily observation for general toxicity, the studies on TAME and ETBE included an FOB to evaluate neuromuscular function and sensory perception. The FOB was performed 1 week prior to the first exposure and after 1, 5, or 20 exposures. All ETBE-exposed animals were evaluated approximately 1 h after the end of exposure. Four TAME-exposed animals were examined approximately 1 h after the end of exposure and 10 animals were examined the following morning in each exposue group. The FOB consisted of an evaluation of the following parameters: Tail Pinch, Rotorod Performance, Body Temperature, Righting Reflex, Auditory Response, Hindlimb Extension, Foot Splay, Grip Strength, Home-Cage Observation, Hand-Held Observation, Open-Field Observation, Extensor Thrust, Catalepsy, Visual Placing, Tactile Placing, Negative Geotaxis, Vision, Eyeblink, and Pupil Response.

4. Clinical laboratory measurements

All rats were fasted for approximately 18 h following the final exposure to TAME or ETBE and anesthetized with sodium pentobarbital. Blood samples were obtained for the following serum chemistry and hematology parameters: Glucose, Creatine Kinase, Alanine Aminotransferase, Aspartate Aminiotransferase, Alkaline Phosphatase, Urea Nitrogen, Sodium, Potassium, Chloride, Total Protein, Triglyceride, Cholesterol, Albumin, Creatinine, Erythrocyte Count, Hemoglobin, Mean Corpuscular Volume, Platelet Count, Total and Differential Leukocyte Count.

5. Postmortem and histopathology assessments

Necropsies were performed on all ETBE exposed rats and 10 of the TAME-exposed rats. The following tissues were weighed and fixed in 10% neutral buffered formalin: Brain, Adrenal Glands, Gonads, Heart, Kidneys, Liver, Lungs, and Spleen. Approximately 31 other tissues were also collected and fixed at necropsy. Only those tissues from the high exposure and control groups were processed for histological examination.

6. Results

Mortality in the rats exposed to 4000 ppm TAME was the most striking difference between TAME and ETBE observed in these studies. Seven of the 24 animals in the TAME high-dose group died on test (four female and three male rats). No rats exposed to 4000 ppm ETBE died during that study.

The deaths were apparently due to severe central nervous system depression. Clinical observations in both the 2000 and 4000 ppm TAME-exposed groups included sedation, coma, ataxia, coldness to touch, ptosis, hyperirritability, hypoactivity and effects on posture. The incidence and severity of effects were greater in the high dose animals. Rats exposure to 4000 ppm ETBE had much milder signs but appeared to be sedated and displayed mild to moderate ataxia during exposure. The ETBE-exposed animals appeared normal 15 min after the end of exposure.

The FOB assessment confirmed the clinical observations. TAME-exposed animals evaluated 1 h after exposure, especially the 4000 ppm

Table 2
Relative organ weights[a] from a 4-week inhalation toxicity study of ETBE or TAME in **male** rats

Organ	Control	500 ppm	2000 ppm	4000 ppm
ETBE				
Fasted BW (g)	355 ± 23.8	353 ± 15.8	358 ± 24.2	358 ± 18.9
Liver	2.86 ± 0.21	2.84 ± 0.15	2.96 ± 0.21	3.32 ± 0.25*
Kidneys	0.77 ± 0.10	0.77 ± 0.03	0.81 ± 0.05	0.86 ± 0.10
Adrenals	0.015 ± 0.001	0.014 ± 0.002	0.014 ± 0.002	0.016 ± 0.002
TAME				
Fasted BW (g)	427 ± 34.3	427 ± 23.1	426 ± 16.4	367 ± 28.1*
Liver	3.13 ± 0.17	3.28 ± 0.49	3.61 ± 0.19*	3.90 ± 0.29*
Kidneys	0.81 ± 0.07	0.80 ± 0.07	0.89 ± 0.07	0.90 ± 0.06*
Adrenals	0.015 ± 0.002	0.017 ± 0.003	0.017 ± 0.002	0.020 ± 0.002*
Testes	0.83 ± 0.10	0.80 ± 0.05	0.83 ± 0.08	1.00 ± 0.07*
Lungs	0.36 ± 0.02	0.38 ± 0.02	0.39 ± 0.09	0.42 ± 0.03*
Brain	0.52 ± 0.04	0.52 ± 0.02	0.50 ± 0.03	0.56 ± 0.03*

Data are mean ± standard deviation, 10 rats/group; $n = 7$ for 4000 ppm TAME-treated group.
[a] Relative organ weight = (organ weight (g)/fasted body weight (g)) ×100.
* Significantly different from controls, $P \leq 0.05$.

group, displayed reductions in tail pinch response, righting reflex and negative geotaxis, along with reduced body temperature, impaired rotorod performance and increased hindlimb splay. The signs of CNS depression were absent in animals examined 18 h after the end of exposure. In animals exposed to 4000 ppm ETBE, the only FOB changes were reduced body temperature in males after the fifth exposure and a significant trend in hindlimb splay in males and females.

Body weight gain was significantly reduced only in male rats exposed to 4000 ppm TAME (Table 2). Mean body weight in this group after 4 weeks of exposure was 14% below control animals. No other TAME- or ETBE-treated groups demonstrated differences in body weight (Table 3).

Exposure to 4000 ppm TAME or ETBE caused an increase in relative liver weights in males and females (Tables 2, 3). Females also had increased relative liver weights at 2000 ppm, as did males exposed to TAME. Differences between TAME and ETBE were most evident in the high dose males in which TAME-exposed animals had relative increases in adrenal, kid-

Table 3
Relative organ weights[a] from a 4-week inhalation toxicity study of ETBE or TAME in **female** rats

Organ	Control	500 ppm	2000 ppm	4000 ppm
ETBE				
Fasted BW (g)	215 ± 9.5	210 ± 3.2	211 ± 3.5	209 ± 8.3
Liver	2.80 ± 0.17	2.93 ± 0.10	3.08 ± 0.22*	3.15 ± 0.24*
Kidneys	0.77 ± 0.07	0.81 ± 0.06	0.81 ± 0.05	0.80 ± 0.05
Adrenals	0.027 ± 0.004	0.027 ± 0.005	0.027 ± 0.003	0.029 ± 0.003
TAME				
Fasted BW (g)	224 ± 10.3	221 ± 13.0	217 ± 14.3	216 ± 13.4
Liver	2.88 ± 0.17	3.03 ± 0.23	3.14 ± 0.15*	3.58 ± 0.22*
Kidneys	0.81 ± 0.07	0.80 ± 0.06	0.83 ± 0.05	0.85 ± 0.05
Adrenals	0.028 ± 0.003	0.027 ± 0.004	0.028 ± 0.002	0.032 ± 0.001*

Data are mean ± standard deviation, 10 rats/group; $n = 7$ for 4000 ppm TAME-treated group.
[a] Relative organ weight = (organ weight (g)/fasted body weight (g)) ×100.
* Significantly different from controls, $P \leq 0.05$.

neys, testes, brain, and lung weights. This group also had a significantly reduced body weight unlike any other treatment group.

No treatment-related histopathologic findings were noted for either compound. Clinical chemistry and hematology findings were absent with ETBE treatment and minimal with TAME. Increased serum cholesterol was found in both male rats (at 2000 and 4000 ppm) and female rats (4000 ppm) exposed to TAME. The 4000 ppm males also had reduced serum triglycerides. A single male rat in the 4000 ppm TAME group had an increase in serum alanine aminotransferase (ALT). This animal also displayed multifocal hepatocellular necrosis that can be associated with elevated ALT. The significance of this finding is unclear as this occurred in only one of the seven animals examined. (Three animals had died on test due to CNS depression.)

7. Discussion

The major findings from the 4-week inhalation toxicity studies on TAME and ETBE were: (1) TAME caused mortality and significantly more CNS depression than ETBE; (2) neither compound caused lasting changes in FOB parameters; (3) both compounds caused increased relative liver weights without histopathology or functional changes; (4) the NOAEL in both studies was 500 ppm.

The mortality observed with TAME at 4000 ppm was unexpected because a 13-week inhalation study with MTBE had been conducted with concentrations up to 8000 ppm without any mortality [5]. Strain differences could have a role as the MTBE study used Fischer 344 rats. However, a 28-day oral toxicity study with TAME [6] reported 20% compound-related mortality in Sprague–Dawley rats in the high dose group of 1 g/kg. These deaths were also attributed to central nervous system depression as there were no gross or histopathology changes to indicate organ-specific tissue injury.

Subchronic inhalation studies with TAME, ETBE, and MTBE have all found increased relative liver weights in rats exposed to 4000 ppm. However, subchronic oral studies with TAME or MTBE [7] did not cause an increase in relative liver weight at doses up to 1 g/kg. The reason for this difference is unknown. Further studies on the pharmacokinetics and metabolism of these ethers are necessary to address this issue.

The 13-week inhalation study with MTBE found increased kidney and adrenal weights that were not seen in the present 4-week ETBE study. The metabolism of ETBE should be similar to MTBE. *O*-Dealkylation of MTBE and ETBE will result in the same metabolite, t-butyl alcohol. The different aldehydes produced by this metabolic step are unlikely to distinguish them toxicologically. The differences in biological responses observed between ETBE and MTBE could be due to the different strain of rat tested, the length of the study, or differences in the pharmacokinetics of the parent compound.

The major metabolite of TAME is likely to be t-amyl alcohol. A 90-day inhalation toxicity study on t-amyl alcohol was done in CD-1 mice, Fischer 344 rats, and beagle dogs [8]. At 1000 ppm, 6 h/day, for 5 days/week, male rats and dogs had increased absolute and relative liver weights. Both species showed signs of central nervous system depression at that concentration. Blood measurements showed that the excretion of t-amyl alcohol was saturated at the 1000 ppm exposure concentration. Mice however had no signs of CNS depression, no organ weight changes, and even at exposure concentrations of 1000 ppm rapidly excreted the alcohol ($t_{1/2} = 29$ min). This study on t-amyl alcohol again points out the importance of understanding the pharmacokinetics and metabolism of these ethers in order to make sound risk assessment conclusions.

Another important factor in evaluating the health risks of oxygenates is the concentration and duration of human exposure. Human exposure to all these ethers will come primarily from refueling or other activities associated with gasoline containing these ethers. The lower vapor pressures of TAME and ETBE make measurements of MTBE exposures a reasonable upper bound for the other ethers. Quantitative studies on exposure have found concentrations

of MTBE in the breathing zone of people filling cars to be <1–4 ppm [9]. Studies done to assess the odor threshold of these ethers in gasoline vapor found that these ethers gave the gasoline a distinctive, typically unpleasant, and more readily detectable odor [10]. The odor thresholds for the ether-gasoline blends were 15–80% lower than gasoline alone. This could be an important factor in understanding the reaction that some individuals experience when refueling with oxygenated gasoline.

Additional toxicology studies on TAME and ETBE are necessary to understand the potential health risks for these ethers. Recently, several manufacturers of TAME signed an Enforceable Consent Agreement with the EPA to conduct such studies [11]. Over the next 3 years more data on TAME's pharmacokinetics and metabolism, genetic toxicity, reproductive and developmental effects, and subchronic toxicity will become available.

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Toxicology Letters

Can chemicals be loved? – A problem for 2000

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Abstract

Public perception of the value of chemicals in daily life depends on an often irrational set of conclusions reached on partial or defective information often derived from single issue pressure groups. Benefits are consistently undervalued, clean water and uncontaminated food are taken for granted and changes in the pattern of disease are assumed to have occurred as a result of other kinds of intervention. If we are to ensure a proper evaluation of the place of chemicals in our lives then we will have to persuade opinion formers of the need for rational debate, inform the scientifically literate part of the population about hazard, risk, the difficult concept of safety and the dangers of mistaking association for causation. Changes in science in the field of toxicology give us a more reasonable ground for this debate than has existed in the past and the way in which these can be exploited will be discussed.

Keywords: Chemical exposure; Safety; Reproduction; Pesticide; Neoplasia

1. Introduction

In a day at home with a notepad, monitoring simple tasks in obtaining and preparing food, cleaning, caring for children or the elderly or repairing defects in the structures which seem constantly to collapse about us, it becomes evident that we depend heavily on the use of a large number of chemicals. To provide the data for appropriate human risk/benefit analysis following the potential exposure to these chemicals we have sought clues from epidemiology, studied the effects of controlled or accidental exposures or used bioassays of various kinds. Uncertainties in extrapolations from bioassays in particular have fuelled concerns about adverse effects of chemicals in the public mind. However, advances in our understanding of the processes which may be disturbed by these exposures now permit different approaches; as widely operating mechanisms affecting fundamental cellular changes become more transparent, risk/benefit analyses of greater rigour may be made. In this way the inappropriate nature of some traditional extrapolations becomes clear, but new uncertainties, some related to individual genetic profiles, become apparent. It is a statement of the obvious that in the selection of hazards for evaluation, public opinion will (and should) be influential but as knowledge grows the need for an informed debate becomes a greater imperative and elusive concepts such as safety are greater and greater obstacles to rational progress. I believe that we have benefited from our use of chemicals and that we can improve the pay-off with time – how can we persuade others of this? A major problem is the differing perspectives which we all have – let us start with safety.

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2. Safety

It is difficult to decide what we mean by 'safe'. Siddall [1] defined it in the following way: "Safety is the degree to which temporary ill health or injury, or chronic or permanent ill health or injury, or death are controlled, avoided, prevented, made less frequent or less probable in a group of people". The concept of a group is enormously important. Responsibility for the prevention of malaria gives you a different perspective on DDT than the one you hold if you look after birds of prey. There is a tendency to ignore the consequences of 'negative' regulatory actions but a ban on the use of herbicides which resulted in the use of more labour and machinery would probably have an adverse outcome in terms of farm workers health; 61 people were killed on farms in the United Kingdom in 1989, mostly by machinery. How do you evaluate the risks/benefits of fruit and vegetables in a diet, the effect of yields on their costs and consumption, the existence of natural toxicants in some untreated foods, the benefit of additives in preventing free radical production – and all these in terms of the individual? I believe we must take a different approach.

If we begin from the human rather than the experimental end of things we can consider some real problems. Lice of several varieties, bed bugs, fleas, mites and ticks all spread disease in man. Leave aside the self-induced problem of trekking holidays and Rocky Mountain spotted fever and consider the day-to-day problem of head lice. These insects are commonly found in schools; most parents will have had to deal with them. The conventional (and effective) treatment is to shampoo the hair with a preparation containing γ-hexachlorocyclohexane (HCH) but concerns about the use of this compound as Lindane in remedial wood treatment in the home have lead to newspaper articles suggesting links between exposure and various blood dyscrasias, notably aplastic anaemia [2]. The Advisory Committee on Pesticides (ACP) of the United Kingdom has examined published and unpublished accounts of cases with apparent association of ill health and HCH exposure and has found no evidence of linkage; examination of a number of large aplastic anaemia registries has failed to show an association of the disease with pesticide exposure [3]. It is interesting to note how this question has been treated by the media, with emphasis on case reports, often without verification of exposure (something which is very difficult to do – think of Agent Orange) and with causation assumed for widely disparate clinical syndromes of differing pathogenesis including aplastic anaemia, Hodgkin's disease, paralysis and epilepsy. Curiously, and in my view typically, these effects are generally attributed to very low exposures of the kind which might obtain in a house treated by professionals or to transiently high exposures in a DIY exponent. The effects of the much greater exposures from anti-louse treatments or to agricultural operators where the compound is used as a spray have been largely ignored. They are seen by the public as being somehow different, just as therapy is seen to differ from passive exposure.

These problems lead us to consider that a more rational approach, requiring a better understanding of mechanism and process, is what is required. It will be a long haul, but we must inform those who may influence the future, including teachers and non-science graduates. It is worth considering how we might argue in particular areas of concern.

3. Reproductive effects

The significance of particular events is always clearer in retrospect but it is clear that for many the Thalidomide tragedy altered thinking about chemicals in our environment. Damage to the embryo and fetus is a major concern to the public and has sometimes led to irrational concerns (about radiation, for example).

It is not reasonable to expect a profound understanding of the mechanisms of embryogenesis in the population at large. The idea of the existence of a blueprint in the genes allowing the construction of an embryo has persisted since the early days of experimental embryology and is still found in the minds of many biologists. No such plan exists; epigenetic factors are of great

significance and an extraordinary parsimony exists in the use of a number of basic processes in building the embryo [4]. That such a complex process ever goes to successful completion is surprising only if you believe in plans; the establishment of a complex body form and the control of the necessary processes depends on a limited number of highly conserved mechanisms; an observation which itself suggests the evolutionary difficulty of establishing them and one which is reinforced by the fact that fetal loss remains the commonest outcome of fertilization. The conservative nature of the developmental process is evident from the use of identical mechanisms of control in most of the Animalia. The reasons for this are explored elsewhere [4,5] but if relatively few mechanisms are involved then our capacity to ensure they are not disturbed by xenobiotics increases and the way in which this approach might be enhanced can now form part of our planning.

Some of the mechanisms are explicable in general terms and the examples given allow us to inform people about the relative importance of risks in this field. Better definition of the way in which the genes influence development tell us something of what processes may be affected by what classes of compound – data we already have for the process of carcinogenesis.

4. Neoplasia

Firstly, there are some myths to be dispelled. Cancer is essentially a disease of the elderly; more than two-thirds of human tumours occur in individuals over 65 years of age. The tumours are not randomly distributed among cell types – some are clearly more susceptible than others. It should not be supposed that cells in cancers are fundamentally different from normal cells; cells in tumours possess no properties not possessed by certain normal cells and their growth rates are comfortably exceeded by many epithelial and bone marrow cells. No tumour grows as fast as the normal conceptus. With the exception of the increase in lung cancer in women and in skin cancer (for which there are good explanations) age-corrected rates for neoplasms are not changing.

The autonomous cell proliferation which characterizes neoplasia may be due to the removal of a growth inhibitory gene product or the induction of a specifically determined growth rate advantage in a cell lineage. Growth factors which are vital in early development of the conceptus are produced by genes (proto-oncogenes) which if altered can overproduce normal gene product or altered product with disastrous consequences for the organism (oncogenes). Highly conserved mechanisms of DNA repair exist and to prevent this type of damage and interference with repair may have catastrophic consequences – as seen in the disease xeroderma pigmentosum. In many human tumours in genetically normal individuals, genes involved in DNA repair and monitoring are altered. An important part of the process is the gene p53 altered in more than half of all human tumours and in more than 80% of cases in some neoplasms. p53, a cell cycle-related DNA binding protein which regulates transcription, acts as a tumour suppressor gene and in many human tumours one allele is mutant and the other deleted [6]. The gene product acts as a cell cycle check point leading to 5-phase delay in cells with genetic damage thus allowing more time for DNA repair before changes are fixed by the next cell division [7]. Levels of p53 appear to be regulated by differential gene expression as well as at the post transcriptional level and there is evidence for a particular role for this gene in differentiation; it has been shown that its level of expression in fetal mice is not well correlated with cell proliferation but up-regulation of p53 gene expression may be necessary to inhibit cell cycle progression and to allow terminal differentiation at many sites [7].

This is an example of specific information related to particular tumour types, others will be illustrated. These advances in our basic understanding have facilitated the evaluation of the role of human exposure to chemicals in carcinogenesis in a different way; we now know where to look for damage. The covalent binding of chemicals or their reactive metabolites to DNA produces adducts and the extent of formation of

these adducts is closely correlated with oncogenicity; as an example, the differences in oncogenicity between isomers of 2,4- and 2,6-dinitrotoluene can be correlated with the extent of adduct formation, the particular adducts formed and their persistence [8]. The methodology of searching for adducts – currently time consuming and technically demanding – is becoming more accessible and the examination of readily available and readily sampled proteins such as haemoglobin offers a field of study which may provide useful population-based data.

But we are further ahead in managing disease. Detailed accounts of the genetic changes in colon cancer, a major cause of death in most developed countries [9], have led to so much better an understanding of the chronology of events as to give rise to proposals for prevention by once-only sigmoidoscopy at 55–60 years with subsequent surveillance for the 3–5% of individuals found to have high-risk lesions [10]. The rationale is that the sequence of events (6—7) necessary to produce large bowel cancer will not have time to occur in individuals if early changes are present at 60 years. The savings over the currently recommended faecal blood/endoscopy regime every 3–5 years are of major significance for the health service, but the knowledge base on which they depend should certainly influence regulators.

5. Exposure data

A difficulty in dealing with possible human exposure levels to low-dose toxicants, natural or synthetic, is that we have relatively little useful information on real intakes; those data we have pose more problems than they resolve. The idea of food-related risks at the 'acceptable' one in a million level seems to me to be to be a poor basis for reassuring the public because it is based on such poor data – in any case these are not properly quantified risks but expressions of mathematical concepts. Ranking of relative risks may be more helpful in this respect and gets us away from the problem of extrapolation from effects produced at high dose in animals to predictions about effects occurring at a low frequency in man. This is a major stumbling block; you will all be familiar with the pervasive concept that if an effect is produced, in animals, at some large multiple of the human dose then there is probably a linear relationship of dose and effect that ensures that no dose is safe for man. This is rarely true, and at small doses becomes improbable – yet it appears to be accepted by many groups despite the fact that most of us accept a threshold concept of dose/effect relationships – how many of the public would volunteer to have an operation after 1/10th of the normal dose of anaesthetic, much less 1/100th or 1/1000th? It is hard to get the public to accept that there can be a dose of a chemical which is without effect where clear toxicity has been demonstrated at high dose, despite some obvious examples.

Intakes and exposures are evaluated differently when related to naturally occurring substances and synthetic materials. We also accept different standards of proof in regulatory matters; herbal remedies do not have to satisfy the same stringent criteria demanded of conventional medicines. This is a matter of convenience and usage, but it has its dangers in this and other fields – some naturally occurring food supplements have proved to be dangerous (see, for example, [11]).

6. Conclusions

Much of the concern which the public has about chemical exposure is irrational. Benefits are consistently undervalued, many significant exposures are ignored, safe water, uncontaminated food, properly preserved food and drink are taken for granted and the role of chemicals in the production of clothing, furniture, paints, kitchen utensils, glass, paper and protection against pests are ignored. Better science provides better information, better protection against accidental injury and hopefully a more rational debate. However, it is for the protagonists of science to defend its value and those who deal with the public will know that we are at a disadvantage. Credibility must be earned by consistency in the defence of rational argument.

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Chlorinated hydrocarbons: estrogens and antiestrogens

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds bind to the intracellular aryl hydrocarbon (Ah) receptor and induce a diverse spectrum of biochemical and toxic responses. Ah receptor agonists also modulate several endocrine pathways, and research in several laboratories has shown that TCDD and related compounds inhibit estrogen (E2)-induced responses in the rodent mammary and uterus and in human breast cancer cell lines. The mechanisms of interaction between the TCDD- and E2-induced signaling pathways are complex and some of the inhibitory effects may be related to 5′-flanking inhibitory-dioxin responsive elements (i-DREs) in target genes. The antiestrogenic and antitumorigenic activity of Ah receptor agonists has been used to prepare a series of relatively non-toxic alkyl polychlorinated dibenzofurans which have clinical potential for treatment of mammary cancer.

Keywords: TCDD; Ah receptor; Antiestrogenicity

1. Introduction

Synthetic organochlorine compounds such as 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p*-,*p*′-DDT), kepone, other organochlorine pesticides and polychlorinated biphenyls (PCBs) were widely used industrial chemicals for diverse applications. In the 1960s, it was recognized that many of these compounds were highly persistent in the environment and preferentially bioconcentrated in the food chain. Moreover, there was evidence suggesting that organochlorine pollutants, such as DDT and related compounds, PCBs and other halogenated aromatics including the polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), may contribute to reproductive problems associated with fish and wildlife populations. Recognition of these potential problems has resulted in modified production, use, handling and disposal of most organochlorine compounds and outright banning of chemicals such as PCBs. *p*,*p*′-DDT, a widely used insecticide, is no longer used in first-world industrial nations; however, this compound is still used for insect control in some countries. Not surprisingly, levels of most persistent organochlorine pollutants are decreasing in fish, wildlife and human populations, and this correlates with increased reproduction in some wildlife species in industrial areas such as the Great Lakes region in North America [1].

Recent articles have discussed the potential role of organochlorine environmental contami-

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nants and other industrial compounds as 'endocrine disrupters' and their role in wildlife reproduction [2,3]. It has been hypothesized that many of these same compounds may play a role in the increased incidence of male reproductive problems and breast cancer in women [4,5]. The validity of these hypotheses has been questioned [6] and further research is required to delineate the role of organochlorine contaminants on human health. The term 'endocrine disrupter' may be confusing since almost all chemical substances at some dose will disrupt one or more endocrine pathways. Most of the organochlorine compounds which have been described as 'endocrine disrupters' are hormone or antihormone mimics that modulate endocrine signaling pathways. For example, several halogenated hydrocarbons bind with moderate to weak affinity to the estrogen receptor (ER) and these include *o,p'*-DDT, kepone, methoxychlor, several cyclodiene-derived insecticides, and hydroxy PCBs (reviewed in [2,3,5]). These compounds are all ER agonists and induce a broad spectrum of ER-mediated responses. In recent collaborative studies (with T. Zacharewski), we have shown that some hydroxy-PCBs are also partial ER antagonists. A recent study [7] reported that 1,1-dichloro-2,2,-bis(*p*-chlorophenyl)ethylene (-*p,p'*-DDE) bound to the androgen receptor; however, both in vivo and in vitro studies with *p,p'*-DDE showed that this compound is an androgen receptor antagonist. Halogenated aromatics also bind to other proteins involved in endocrine response pathways; hydroxylated PCBs, PCDDs and PCDFs bind to transthyretin, the major thyroxine transport protein in rats [8], and it has been suggested that other halogenated biphenyls and diphenylethers may bind proteins involved in thyroid hormone action [9].

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatics are industrial by-products which have also been detected in the environment. These compounds induce a diverse spectrum of biochemical and toxic responses in laboratory animals and mammalian cells in culture [10,11]. Poland and coworkers [12] first characterized the hepatic cytosolic aryl hydrocarbon (Ah) receptor binding protein in C57BL/6 mice and subsequent studies have identified this protein in both TCDD-responsive and non-responsive tissues and organs. The mechanism of action of Ah receptor-mediated responses has been extensively investigated and the essential steps in this mechanism have been derived from initial studies on induction of CYP1A1 gene expression [12–16]. The unbound cytosolic Ah receptor complex is associated with heat shock protein (hsp) 90 and initially binds to TCDD forming a 270- to 300-kDa complex. This complex undergoes loss of hsp 90 to form a nuclear heterodimer with the Ah receptor nuclear translocator protein (Arnt); the interaction of this complex with distal enhancer sequences or dioxin/xenobiotic responsive elements (DREs/XREs) results in transactivation of the CYP1A1 gene. The induction of other Ah receptor-mediated responses may also incorporate a similar mechanism; however, it is clear that many other factors also regulate the age-, sex-, strain- and species-specific effects observed for TCDD and related compounds. Based on gene cloning experiments [17–19], the Ah receptor and Arnt proteins have been identified as members of the basic helix-loop-helix family of nuclear proteins and are the only members of this family which are ligand-induced transcription factors (LTFs). It should also be noted that the endogenous ligand for the Ah receptor is unknown.

2. TCDD and related compounds – interactions with other endocrine response pathways

TCDD and related halogenated aromatic hydrocarbons bind to the Ah receptor and their affinity for other hormone receptors has not been reported. Nevertheless, TCDD and other Ah receptor agonists disrupt many other endocrine response pathways and these effects may play an important role in development of Ah receptor-mediated toxic responses (reviewed in [20]). For example, it has been shown in laboratory animal studies that TCDD alters thyroid hormone levels in rodents, decreases testicular and adrenal steroidgenesis, modulates ACTH-induced responses in rats, decreases melatonin levels in rats and

levels of several steroid hormone receptors including the estrogen and glucocorticoid receptor. TCDD also decreases epidermal growth factor (EGF) receptor in several target organs/cells and modulates expression of cytokines and growth factors which play a role in several endocrine response pathways. The effects of TCDD and related compounds on reproduction have been extensively documented [21], and it is likely that Ah receptor-mediated modulation of endocrine response pathway plays a role in these and other toxic responses. The mechanisms associated with these induced toxicities are currently unknown.

3. Ah receptor-mediated inhibition of estrogen (E2)-induced responses

Kociba and coworkers [22] first reported that in long-term feeding studies with TCDD, there was a dose-dependent decrease in spontaneous mammary and uterine tumors. Subsequent studies by Gallo, Gierthy, Safe and coworkers demonstrated that TCDD inhibited several 17β-estradiol (E2)-induced responses in the rodent mammary and uterus and in human breast cancer lines [23–26] (Table 1). TCDD inhibited age-dependent formation of mammary tumors and growth of 7,12-dimethylbenz[*a*]-anthracene (DMBA)-induced mammary tumors in female Sprague–Dawley rats and mammary tumor growth in immunosuppressed B6D2F1 mice bearing MCF-7 cell xenografts and treated with E2.

TCDD also inhibited the following E2-regulated responses in female Sprague–Dawley rat uteri: cytosolic and nuclear ER and PR binding; peroxidase activity; EGF receptor binding and mRNA levels; and c-*fos* protooncogene mRNA levels. Some of the same E2-induced responses were also inhibited in mouse uteri. The antiestrogenic activities of TCDD and related Ah receptor agonists have been extensively investigated in ER-positive MCF-7 and T47D human breast cancer cell lines and inhibition of the following E2-induced responses have been reported (Table 1): cell proliferation; postconfluent focus production; secretion of tissue plasminogen activator activity, 34- (cathepsin D), 52- (procathepsin D) and 160-kDa proteins; PR binding and immunoreactive protein; glucose-lactate conversion; cathepsin D, pS2 and PR mRNA levels. TCDD also inhibited growth factor-induced cell proliferation in these same cells [20]. Mechanistic studies suggest that formation of the nuclear Ah receptor is required for TCDD-induced antiestrogenicity and these inhibitory responses can be observed in the absence of induced E2-metabolism. Fig. 1 illustrates several possible mechanisms associated with the antiestrogenic effects of Ah receptor agonists and these include: direct interaction between the nuclear Ah receptor and *cis*-acting genomic

Table 1
Antiestrogenic activity of TCDD and related compounds in the rodent and human breast cancer cell lines

		Response
A.	Rodent mammary tumors	Inhibition of spontaneous mammary tumor formation in Sprague–Dawley rats
		IInhibition of transplanted tumor growth in athymic nude mice
		Inhibition of DMBA-induced tumor growth in rat
B.	Rodent uterus	Inhibition of E2-induced uterine wet weight peroxidase activity and progesterone receptor (PR) binding
		Inhibition of E2-induced EGF receptor and for mRNA levels
		Downregulation of uterine ER levels
C.	Human breast cancer cell lines	Inhibition of E2-induced proliferation and postconfluent focus production
		Inhibition of E2-induced secreted proteins (cathepsin D, procathepsin D, t-plasminogen activator, pS2, 160-kDa protein
		Inhibition of E2-induced PR, lactate dehydrogenase
		Inhibition of E2-induced gene expression (PR, pS2, cathepsin D)
		ER downregulation

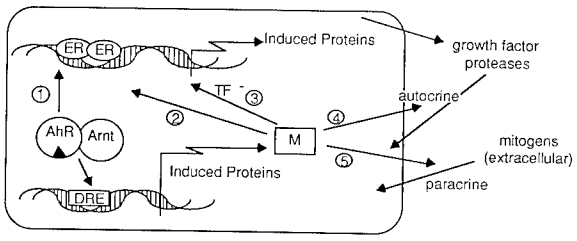


Fig. 1. Inhibition of E2-induced pathways by TCDD and related compounds.

sequences in flanking regions of E2-regulated (pathway 1) and/or growth factor-regulated genes (pathways 4 and 5), induction of *trans*-acting factors or proteins (M) which facilitate degradation of the nuclear ER (pathway 2), or inhibition of E2/mitogen-induced proliferation of breast cancer cells (pathways 3 through 5). Induced metabolism of E2 (pathway 2′) also plays a role by decreasing cellular E2 levels.

Recent studies in this laboratory have investigated the molecular biology of E2-induced cathepsin D gene transcription and inhibition of this response by TCDD and related Ah receptor agonists in breast cancer cell lines [25]. E2-induced secretion of procathepsin D, increased intracellular levels of this protein, and induced cathepsin D mRNA levels. The rate of E2-induced cathepsin D mRNA levels was also determined in nuclear run-on assays. In MCF-7 cells cotreated with E2 plus TCDD, all of the E2-induced responses were inhibited. Moreover, in nuclear run-on assays, the rate of E2-induced cathepsin D gene expression was significantly inhibited within 30 min after addition of 10^{-9} M TCDD. These results indicate that the effects of TCDD are rapid, suggesting that mechanisms which involve induced gene transcription (Fig. 1, pathways 2 through 5) may not be important. Subsequent studies in this laboratory [27] identified an ER-Sp1-like sequence [GGGCGG(n)$_{23}$ACGGG] in the non-coding strand of the cathepsin D promoter (−199/−165). Electromobility shift assays of nuclear extracts from MCF-7 and HeLa cells confirm that both the ER and Sp1 proteins bind to ^{32}P-labeled ER/Sp1 oligonucleotide. Moreover, E2 induced chloramphenicol acetyl transferase (CAT) activity in MCF-7 cells cotransfected with a human ER expression plasmid and a plasmid containing an ER/Sp1 sequence cloned upstream to a thymidine kinase promoter and a CAT reporter gene. In contrast, E2 did not induce CAT activity in MCF-7 cells transfected with plasmids containing mutations in the ER or Sp1 segments of the ER/Sp1 oligonucleotide, thus confirming that both cognate binding sites are required for E2-responsiveness. Using the ER/Sp1-CAT plasmids and constructs containing more extensive 5′-flanking sequences from the cathepsin D gene, it was shown that TCDD inhibited E2-induced reporter gene activity in transient transfection assays. Moreover, in gel mobility shift assays using extracts from MCF-7 cells treated with E2 plus TCDD, it was observed that within 1 h after addition of TCDD, the retarded ER/Sp1 band was not formed. The same effect was observed after incubating nuclear extracts from E2-treated cells with cytosolic Ah receptor transformed with TCDD. These results suggested that the Ah receptor heterodimer directly disrupted the ER/Sp1 complex. Zacharewski and coworkers first reported that an inhibitory-DRE (i-DRE) sequence plays a role in the inhibition of E2-induced pS2 gene expression ([28] and unpublished results). Subsequent examination of the ER/Sp1 oligonucleotide in the cathepsin D 5′-region showed that a GCGCGTG sequence, which is an imperfect XRE, was located between the ER and Sp1 genomic binding sites. The results of several electromobility shift and transient transfection assays confirmed that this i-DRE is responsible for the inhibitions of E2-induced cathepsin D gene expression by TCDD, and this represents a unique Ah receptor-mediated pathway for modulating gene expression and for interaction with another endocrine response pathway.

Tamoxifen and other antiestrogens used for treatment of breast cancer bind to the ER and inhibit E2-induced responses primarily through interactions with this receptor. In contrast, Ah receptor agonists are antiestrogenic via interaction between two different signaling pathways. Research in this laboratory has focused on development of Ah receptor agonists with potential

for clinical treatment of mammary cancer. 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) and related 6-alkyl substituted analogs were originally synthesized for investigating their activities as partial Ah receptor antagonists. MCDF competitively bound with moderate affinity to the cytosolic Ah receptor but was a relatively weak agonist for several Ah receptor-mediated biochemical and toxic responses including induction of CYP1A1 and CYP1A2 in rats or cells in culture; however, in cotreatment studies with TCDD, MCDF inhibited TCDD-induced CYP1A1, CYP1A2, teratogenicity, porphyria and immunotoxicity in a concentration-, organ-/cell-, and species-specific manner. In contrast, MCDF did not inhibit TCDD-induced antiestrogenicity but acted as an Ah receptor agonist for this response. A comparison of the antiestrogenic activity of MCDF and TCDD in the female rat uterus showed that MCDF was approximately 300–570 times less active than TCDD as an antiestrogen [29]. In contrast, MCDF was relatively non-toxic in the rat and, based on enzyme induction studies, TCDD was >158 000 times more potent than MCDF. Similar results were observed in breast cancer cell lines indicating that MCDF and related compounds were relatively non-toxic for the typical Ah receptor-mediated toxic responses but were relatively active as antiestrogens [30]. Preliminary studies indicate that MCDF inhibits growth of DMBA-induced mammary tumors in the female Sprague–Dawley rat and further research on the antitumorigenic activity of MCDF and related alkyl PCDFs is in progress.

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Feminized responses in fish to environmental estrogens

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Abstract

Effluent from sewage-treatment works entering British rivers contains an estrogenic chemical, or mixture of chemicals, that stimulates vitellogenin synthesis in male fish. If the effluent constitutes a significant proportion of the flow of the river, lengthy stretches of entire rivers can be estrogenic to fish. The chemical, or chemicals, responsible for this feminizing effect have not yet been identified. However, many man-made chemicals known to be estrogenic to fish (and other vertebrates) are present in effluent, although which of these, if any, is responsible for the effects noted when caged fish are placed in rivers is unclear presently. In laboratory studies, exposure to estrogenic alkylphenolic chemicals caused a reduction in the rate of testicular development in trout undergoing sexual maturation.

Keywords: Estrogenic chemicals; Vitellogenin; Fish; Aquatic ecotoxicology

1. Introduction

Recently it was reported [1] that when caged male trout were placed in the effluent of sewage-treatment works, they were induced to synthesize vitellogenin. Vitellogenin, the precursor of yolk, is normally produced only in females in response to estrogens from the ovary. Male fish do not normally synthesize vitellogenin [2], because they have very low (often undetectable) concentrations of circulating estrogens. However, male fish respond to exogenous estrogens, administered by injection or via the water, by synthesizing vitellogenin [1,3] in the same way that females do. Thus, the presence of vitellogenin in the plasma of male fish can be used as a biomarker of exposure to estrogenic chemicals [4].

In the last few years, considerable concern has been expressed about consequences to wildlife and humans upon exposure to endocrine-disrupting chemicals [5]. For example, it has been suggested that the developmental abnormalities of the gonad and abnormal sex hormone concentrations observed in juvenile alligators from Lake Apopka, Florida, were caused by contamination of the lake by an extensive spill of pesticide [6]. Our work on fish appears to be one piece of a jigsaw implicating various persistent man-made chemicals in disruption of the endocrine system, which will likely be manifest in many ways.

2. Estrogenic activity in rivers

Our initial work [1] was concerned with effluent; caged male fish placed in the effluent of all sewage-treatment works tested, which were located throughout England and Wales, responded by synthesizing vitellogenin in a very rapid and pronounced manner. The only viable expla-

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nation appears to be that the effluent contains a chemical, or mixture of chemicals, that is estrogenic to fish.

The effluent from sewage-treatment works can constitute a significant proportion of the flow of a river. In some cases, particularly when slow-flowing rivers pass through urban areas (where there are often a number of large sewage-treatment works), the effluent can contribute the majority of the flow; figures as high as 80% or 90% of the water in the river coming from sewage-treatment works are reached in periods of low rainfall. Thus, it seemed possible that not only neat effluent, but also sections of some rivers, might be estrogenic.

To assess this possibility, we placed caged male fish at various locations downstream of sewage-treatment works, left them there for 3 weeks, and then determined their plasma vitellogenin concentrations. In the rivers of high water quality, the estrogenic activity of the effluent was lost within a few hundred metres downstream of where the effluent entered; that is, the river itself was to all intents and purposes not estrogenic (Harries et al., unpublished data). In contrast, in rivers of low water quality, significant stretches of river (many miles) were strongly estrogenic, and in one case essentially the entire length of a river was estrogenic (albeit fairly weakly so in most places) (Harries et al., unpublished data).

Obviously the amount of effluent entering a river, the size of the receiving river, the quality of the effluent, the weather conditions at the time, and probably also other factors, will all influence whether or not a river is estrogenic, and to what degree. Nevertheless, it is obviously the case that the problem of estrogenic activity is not confined to effluent only, but extends to significant stretches of some rivers. The possible consequences of this contamination to aquatic biota are discussed later.

3. Identification of estrogenic chemicals in the aquatic environment

The effluent from all sewage-treatment works tested has been strongly estrogenic to fish [1]. These sewage-treatment works were of different types, and received input of varying composition; all received primarily domestic effluent with varying proportions of industrial effluent of (presumably) changeable composition. Because all effluents proved estrogenic, it suggests (but no more) that it is likely to be the domestic part of the influent that is responsible for the estrogenic activity of the effluent; either the domestic influent is already estrogenic, or it is degraded in the sewage-treatment works to one or more estrogenic chemicals.

There are two distinct approaches that could be taken when attempting to identify the estrogenic chemicals in effluent. One is to make an informed guess, based on existing knowledge of what chemicals are estrogenic, and then try to identify this chemical (or chemicals) in effluent. The other is to make absolutely no presumptions about the identification of the chemical (or chemicals) responsible, and to search with an open mind, combining analytical chemistry with biological assaying in an attempt to isolate and ultimately identify the chemical(s) responsible for the estrogenic effects on fish. We have taken both approaches. Both ultimately need to be followed up by in vivo laboratory experiments, where the chemical(s) identified as contributing to the estrogenic activity of effluent are tested to ensure that they do, in fact, produce estrogenic effects in fish.

Taking the first approach, chemicals known to be estrogenic and present in the aquatic environment include some organochlorine pesticides, some polychlorinated biphenyls (PCBs), some alkylphenolic chemicals, bisphenol-A, some phthalates, and many natural and synthetic estrogens [7,8]. The list of estrogenic chemicals known to be present in the aquatic environment is growing rapidly; a recent report found that half of the chemicals selected randomly from a list of those known to be present in sewage effluent possessed estrogenic activity [9]. As more chemicals are investigated, the number of those shown to possess estrogenic activity is likely to increase; this will make it increasingly difficult to make an informed guess as to which chemical(s) on the list are likely to be responsible for the estrogenic activity of effluent.

A second problem associated with this approach to identifying the main estrogenic chemicals in effluent relates to their estrogenic potency. Essentially all of the estrogenic chemicals known to be present in effluent are very weak estrogens (for example, nonylphenol is four or five orders of magnitude less potent than the natural estrogen 17β-estradiol), and hence they need to be present at reasonable concentrations (micrograms/litre or more) if they are to be significant contributors to the overall estrogenicity of effluent. Such concentrations are readily measurable with existing analytical chemistry techniques [8,10]. The problems will arrive if the chemical responsible for most or all of the estrogenic activity of effluent is not a weakly estrogenic one present in reasonable concentrations, but instead is strongly estrogenic, and hence would need to be present at only very low concentrations to produce the observed effects on fish. One such possibility is ethinyl estradiol; this is the main active ingredient of the oral contraceptive pill, and hence theoretically it could enter sewage-treatment works in domestic effluent. In fact, it has been reported to be present in effluent and rivers at a few nanograms per litre [11], although this possibility needs to be confirmed by good analytical chemistry. Ethinyl estradiol is certainly a very active estrogen in fish (as well as mammals); as little as 1 ng/l causes a rapid and pronounced synthesis of vitellogenin in male fish [1]. Thus, only a very low concentration of ethinyl estradiol, or any chemical as active, in effluent would be required to stimulate vitellogenesis in exposed fish. Such a scenario will be very difficult either to prove or disprove unequivocally; it will require very good analysis of effluent or river water combined with a sensitive, robust and precise technique to identify estrogenic activity.

To date, although we know that some chemicals present in effluent and rivers are estrogenic, we do not know whether it is these chemicals, or alternatively as yet unidentified ones, which contribute most or all of the estrogenic activity present in the aquatic environment. Thus, the second approach described above (which makes no presumptions) is probably the most appropriate way to identify the estrogenic chemicals in effluent and river water that cause the biological effects. We are presently taking such an approach.

4. Physiological effects of estrogenic water on fish

Estrogens have very diverse effects; for example, besides their roles in controlling reproduction, they affect growth and metabolism. However, their major roles are concerned with reproduction, especially in females. Thus, in female fish they control the synthesis not only of vitellogenin, but also the egg-shell proteins [12] and help to regulate gonadotrophin secretion [13]. In males, the role(s) of estrogens remain unclear.

Surprisingly, in view of the fact that it appears that estrogens play their major roles in females, very little attention appears to have been directed so far towards investigating the effects of exposure to environmental estrogens on any aspect of physiology in female fish. In contrast, most attention has been focused on males, particularly with regard to whether exposure to environmental estrogens affects development of the testes, and hence sperm quantity and quality. Although relatively little information has been published to date, it has been shown that controlled exposure to estrogenic chemicals (ethinyl estradiol and a number of alkylphenolic chemicals were tested) leads to both vitellogenin synthesis and attenuated growth of the testes during sexual maturation [3]. The consequences to male fish of vitellogenin synthesis and its accumulation in the blood are unknown; it may be of no consequence, or it may be deleterious – certainly very high concentrations of vitellogenin are associated with kidney failure and increased mortality rates [14]. It seems likely that a reduced rate of testicular growth would reduce fertility, but although this seems obvious, it has yet to be demonstrated.

The limited results published to date suggesting that exposure to estrogenic chemicals could have deleterious consequences [3] have been obtained using relatively short exposure times of a few weeks. In the 'real world', in at least some

situations, fish would be exposed throughout their lives to estrogenic chemicals (albeit possibly at low concentrations). The consequences, if any, of such prolonged exposure are unknown. However, it is wise to recall that the issue of estrogenic contamination of the aquatic environment was highlighted by the finding of hermaphrodite fish in some British rivers [4], suggesting that there are real, and important, consequences to aquatic biota when they are exposed to water containing endocrine-disrupting chemicals. The incidence of hermaphroditism in fish in British rivers (or rivers anywhere) is unknown, but is being actively investigated presently in a number of countries.

Hermaphroditism is probably caused by exposure to endocrine-disrupting chemicals at a very early stage in the life cycle of a fish. Much detailed work, undertaken to produce monosex populations of fish for the aquaculture industry [15], has demonstrated that there is a narrow window, comprising about 10 days either side of hatching, when sex is labile, and exposure to hormones can affect sex differentiation. Thus, it is possible that exposure of eggs or juveniles, even for a relatively brief period, to estrogenic chemicals (or to chemicals which mimic other steroids, or antagonize them) at this particularly sensitive time could modify subsequent sexual development. Research is underway to assess whether exposure of eggs and/or juveniles does indeed affect subsequent sexual development.

5. Bioaccumulation of estrogenic environmental chemicals

Many of the estrogenic chemicals present in the aquatic environment are hydrophobic, and hence have a strong tendency to bioconcentrate and bioaccumulate (up the food chain) in aquatic organisms. For example, the bioconcentration factors (BCF) for many organochlorine pesticides, such as DDT, are between 1000 and 100 000. Similarly, the BCF of nonylphenol in fish is probably around 300 [16]. This factor is obviously extremely important when trying to assess the significance of contamination of the aquatic environment by estrogenic chemicals. Whatever the concentration of a particular chemical in the water, if that chemical does not enter the fish to any significant extent (i.e. the BCF is much less than 1), then it is extremely unlikely to cause any effects. In contrast, a chemical could be present at a very low concentration, but if it bioconcentrates to a high degree, then it could be physiologically very important.

Further, not only does one need to know what gets into the fish, and to what degree, but it is also important to know whether that chemical is metabolized in the fish, to what, and where within the fish the chemical and its metabolite(s) are located. For example, an estrogenic chemical might accumulate in adipose tissue (because many are lipophilic), but not at a concentration high enough to exert any significant physiological effects. However, during ovarian development, when lipid reserves are mobilized and stored in oocytes, the estrogenic chemical might also be mobilized, and accumulate and concentrate in oocytes. When the oocytes are ovulated and fertilized, the estrogenic chemical would be present during the critical period when sex differentiation occurs.

The very variable degree of bioconcentration of xenobiotics poses considerable problems when attempting to identify the chemical, or chemicals, in effluent causing the 'feminization' of fish. As already discussed, a combination of chemical analysis and bioassay can be used to identify the main estrogenic chemicals in effluent. However, such an approach may not necessarily identify the chemical that is causing the effects in fish, if this chemical contributes only a small proportion of the estrogenic activity of the water, but has a very high BCF compared to the other estrogenic chemicals in the water.

6. The 'mixtures issue'

This is a complex but very important issue. It is unlikely that fish (or any other aquatic biota) are exposed to only one estrogenic chemical at any time; rather, the water is likely to contain a mixture of chemicals possessing estrogenic activity. It is possible that the effect of one chemical

may be negated by an antagonistic effect of another chemical. Alternatively, the effects could be cumulative, or even synergistic. In an attempt to begin addressing this issue, it has been shown that a mixture of different weakly oestrogenic pesticides produces a greater effect than if each is tested individually [17], and likewise a mixture of pesticides, a plasticizer, and alkylphenols produced an enhanced estrogenic effect, compared to when each estrogenic chemical was tested individually [4].

The issue becomes more complex when it is realized that the aquatic environment is likely to contain not only estrogenic chemicals, but also anti-estrogenic ones (some chemicals can, of course, function as both agonists and antagonists, depending on the circumstances). Yet another level of complexity is reached if chemicals that act on other steroid receptors (such as the androgen receptor or the progesterone receptor) are also present. Already it has been shown that the major persistent metabolite of DDT, *p,p'*-DDE, is a potent androgen receptor antagonist [18]. Thus, whereas one metabolite, *o,p'*-DDT, is estrogenic, another metabolite is anti-androgenic. Many of the chemicals reported to be weakly estrogenic, such as the alkylphenols, are each very heterogeneous mixtures of different isomers, and hence it is likely that, even within a single chemical, different isomers will have different effects via different mechanisms.

7. The way forward

Despite the considerable amount of attention devoted recently to the possible effects of exposure to endocrine-disrupting chemicals (particularly estrogenic chemicals), there is, in fact, presently very little, if any, unequivocal evidence that wildlife are being adversely affected by exposure to such chemicals. A major priority is to gather such evidence, if it exists; the presence of reproductive problems in alligators in Florida, birds in a number of locations in the U.S. and Canada, and fish in the UK, suggests that problems do exist. Identifying the causes of these problems will be very difficult (there may be different causes to different problems in different situations). Collaborations between analytical chemists and physiologists will be required if progress is to be made. It will be necessary to identify not only chemicals of interest in the water, but also within the animal. It will also be necessary to understand how these chemicals are metabolized within the animal of interest. Even then, the presence of numerous different chemicals, with many varied mechanisms of action, will make it extremely difficult to provide clear answers to the many questions being posed presently.

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Developmental effects of dioxins and related endocrine disrupting chemicals[1]

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Abstract

Alteration of hormones has long been known to affect development. TCDD and related PHAHs modulate the levels of many hormonal systems. Dioxins cause a spectrum of morphological and functional developmental deficits. Fetotoxicity, thymic atrophy, and structural malformations are often noted. Delayed genitourinary tract effects have been observed, and recent studies reported behavioral effects. Highly exposed human offspring have exhibited developmental problems as well. Recently, hormonal and neurological abnormalities have been reported in infants from the general population. The complex alteration of multiple endocrine systems is likely associated with the spectrum of adverse developmental effects caused by dioxin and related compounds.

Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD); Dioxins; Polychlorinated biphenyls (PCBs); Developmental toxicity; Endocrine disruption; Receptor-mediated toxicity

1. Introduction

Development is a highly integrated process which begins with gametogenesis, proceeds with fertilization, embryogenesis, maturation, and eventually senescence. Developmental effects have traditionally been thought of as birth defects; however, it is critical to go beyond this concept to include the entire life cycle. Development is under hormonal control. A precise integration of multiple endocrine systems is required for all stages in development. The role of estrogens, progestins, gonadotropins, androgens, etc. is well known in regards to gametogenesis and development. The brain, endocrine organs, reproductive organs, and peripheral tissues all contribute to proper functioning of the organism throughout its life cycle.

Given that multiple hormones play critical roles in all aspects of development, it is likely that exogenous environmental chemicals, which can mimic, block, or modulate the endogenous chemical messengers can cause developmental effects [1]. The most important family of such environmental contaminants, for which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) is the prototype, is the polyhalogenated aromatic hydrocarbons (PHAHs), which have been shown to be developmental and reproductive toxicants in multiple species (for recent reviews see [2,3]). Many of these compounds, those which are halogenated in at least four lateral positions, have a common mechanism of action which involves binding to a cytosolic protein, the Ah receptor [4]. This protein functions as a ligand-

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activated transcriptional enhancer [5]. Although it is thought of functioning in an analogous manner to steroid receptors, the Ah receptor is a member of the basic helix-loop-helix (bHLH) family of proteins [6]. As with the steroid receptors, the Ah receptor exists in a multi-protein complex in association with heat shock and other proteins. Upon binding to the ligand, a conformational change results in release of the other proteins and association of the ligand binding subunit, the Ah receptor, with another bHLH protein, ARNT. The ligand-bound heterodimer binds to specific sequences in regulatory regions of DNA, leading to alterations in gene expression. A second method to alter cell signalling has recently been proposed involving activation of protein tyrosine kinases [7]. A schematic of Ah receptor action indicating how ligand binding can alter proliferation and differentiation via two mechanisms is shown in Fig. 1.

A receptor-mediated mechanism of action is characteristic of all hormones, growth factors, and cytokines. It is therefore appropriate to consider dioxin and related chemicals which act via the Ah receptor as environmental hormones. In fact, dioxins are potent mimetics, blockers, and modulators. Almost every hormone system examined has been shown to be altered by dioxin in some cell-type, tissue, or developmental stage [5]. Dioxin may be anti-estrogenic, or require

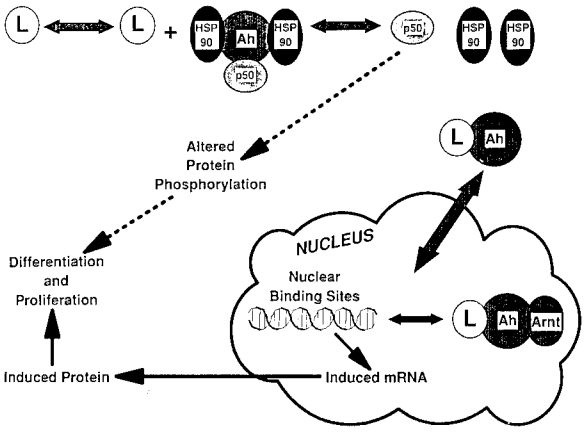


Fig. 1. Schematic of Ah receptor mechanism. L, ligand (TCDD, other dioxins, certain PCBs, etc.); Ah; Ah receptor; HSP90; heat shock protein 90; p50, representative of additional peptides present in multimeric Ah receptor complex; Arnt, aryl hydrocarbon nuclear translocator.

estrogen for its actions. Its effects can resemble both hypo- and hyperthyroidism. Depending on the system, dioxin can antagonize or synergize with glucocorticoids. In addition, dioxin can modulate the actions of insulin, retinoids, growth factors such as TGFα and β, and cytokines such as IL1β. Dioxin can also alter the metabolism of multiple hormones via its induction of both Phase I and Phase II biotransformation enzymes. For example, T_4 is eliminated more rapidly due to induction of glucuronyl transferase activity.

Not all of the PHAHs act through the Ah receptor (Table 1). While Ah receptor interaction appears necessary for all of the laterally substituted dioxins, dibenzofurans, and PCBs, many of the PCBs have difficulty in attaining a coplanar conformation and have extremely limited or no ability to bind to the Ah receptor. In fact, there may be multiple classes of PCBs which have independent, but potentially overlapping, mechanisms and responses [8]. Thus, some of the di-ortho substituted PCBs have phenobarbital-like activity in terms of enzyme induction. Certain PCBs, and especially some of the *p*-hydroxylated metabolites, have estrogenic activity. Sulfonated metabolites have been shown to affect pulmonary function. Some of the lower chlorinated PCBs may inhibit tyrosine hydroxylase activity leading to changes in dopaminergic systems [9] or to changes in calcium homeostasis [10]. Both dioxin and non-dioxin-like PCBs can alter thyroid status, by altered kinetics of thyroid hormones [11] as well as potential interactions with the nuclear thryoid receptor [12]. Recent studies in both humans and rodents have suggested that PHAHs may alter vitamin K metabolism, leading to an increase in bleeding problems in newborns [13]. Prenatal exposure to PCBs has been shown to alter retinoid status [14].

2. Teratogenic effects of TCDD

TCDD and other Ah-receptor ligands are potent developmental toxicants in multiple species (reviewed in [3]). At doses which are not overtly toxic to the mother, dioxin can cause fetotoxicity in the offspring. This is manifested by decreased growth, hemorrhage, and fetal death. Edema is observed in certain species. In

Table 1
PCBs and PCB mixtures

Mixture	% Chlorine	Congener	IUPAC number
Aroclor 1016	21	Dioxin-like:	
1221	21	Coplanar –	
1242	42	3,3',4,4'-Tetrachlorobiphenyl	77
1248[a]	48	3,3',4,4',5-Pentachlorobiphenyl	126
1254[a]	54	3,3',4,4',5,5'-Hexachlorobiphenyl	169
1260	60	Mono-orthocoplanar –	
		2,3',4,4',5-Pentachlorobiphenyl	118
Clophen A30	32	Non-Dioxin-like:	
		2,4,4'-Trichlorobiphenyl	28
		2,2',4,4',5,5'-Hexachlorobiphenyl	153

[a] Mixtures with most dioxin-like congeners.

addition, hypoplasia of the lymphoid organs is often observed at relatively low doses. A recent study [15] has demonstrated that prenatal exposure to PCB #118, which has limited ability to bind to the Ah receptor, leads to hypoplasia of the gastric-associated lymph nodes. Some of the effects on the immune system have recently been shown to be extremely persistent [16]. As previously suggested in studies of prenatal effects on the immune system in mice, thymocyte differentiation is altered in mice prenatally exposed to dioxin [17].

The teratogenic effects of dioxins have been most extensively studied in mice. In fact, as far as obvious structural defects are concerned, until recently there was little evidence of dioxin causing such terata in any other mammalian species [3]. Of course, adverse structural abnormalities induced in birds and fish have been well documented [2]. Dioxin causes hydronephrosis and cleft palate in mice at doses below where any overt fetal or maternal toxicity is detected. Kidney defects have been observed in prenatally exposed hamsters, but these only occur at doses where fetotoxicity is evident. The same can be said for cleft palate in rat pups. In vitro cultures have demonstrated that the embryonic mouse palate is approximately 200 times more sensitive to dioxin-induced clefting than are palatal shelves from rats or humans [18]. Dioxin causes cleft palate by altering the proliferation and differentiation of the medial epithelial cells of the developing palate. These changes are brought about by changes in the balance of various growth factors and their receptors in the target tissue such as TGFα, EGF, and the EGF receptor, and TGFβ1, 2, and 3 [19]. The changes in proliferation and differentiation in the developing palate are also evidenced in the developing urinary tract which inappropriately proliferates leading to blockage of urinary outflow resulting in hydronephrosis in treated offspring (reviewed in [3]). Altered differentiation induced by dioxin has also been reported in the preimplantation embryo [20]. The presence of the Ah receptor has been demonstrated in 8-cell embryos [21]. Both the Ah receptor and ARNT have been shown to be present in the developing palate [22,23] and urinary tract [24]. Doses of dioxin which are teratogenic can be measured in target embryonic tissues within 30 min of maternal exposure [25].

3. Reproductive effects of TCDD

Although dioxin has long been known to be a reproductive toxicant, until recently little effort has been expended in understanding the mechanism of its effects on fertility and reproduction. In part, this has been a result of the focus on overt structural malformations; certain of the effects are also not evident until puberty or even later. Murray et al. [26] demonstrated impaired reproduction in a three-generational study using 10 ng TCDD/kg/day in rats. Recent studies in the laboratory of Peterson and coworkers have examined the effects of prenatal and lactational exposure to TCDD on male rat pups (reviewed in [2]). They noted demasculinization and feminization of the male pups following a single

treatment of 1 μg TCDD/kg on gestation day 15, towards the end of organogenesis. Some of the effects, such as reduced sperm count persisted throughout adulthood. The behavioral changes, originally hypothesized to be due to altered estrogenic or androgenic status, may be due to peripheral effects on the secondary sex structures [27,28]. Based on cross-fostering studies [29], other than the effects on feminizing sexual behavior, decreases in accessory sex organ weights and sperm count, and delayed puberty appear due to prenatal exposure. Similar effects on prenatally exposed male rats and hamsters have been reported by Gray et al. [30]. The developmental exposure to TCDD permanently alters reproductive function in the male offspring of both of these species without any effect on androgenic status. Gray and coworkers failed to observe any change in testosterone or androgen receptor levels in the sex accessory glands or epididymis in the perinatally exposed pups. Nevertheless, the reduction in epididymal and ejaculated sperm appears to be permanent. Although Bjerke and coworkers [27] failed to observe any effect on brain estrogen receptor binding or sexually dimorphic nuclei, CNS involvement has been recently demonstrated by a permanent change in core body temperature induced by this exposure regime in both rats [31] and hamsters (C. Gordon, personal communication).

Gray and Ostby [32] have also examined the effects of prenatal and lactational exposure to TCDD on female rat offspring. In addition to puberty being delayed, structural malformations were present in the external genitalia of the pups. A persistent thread of tissue existed across the vaginal opening in conjunction with partial clefting of the phallus. Because of these alterations, first matings were difficult and often resulted in vaginal bleeding. Ovarian weight was also permanently reduced. Pups prenatally exposed on gestation day 8, rather than 15, had a lower incidence and severity of the genital malformations, but exhibit premature reproductive senescence and an early decline in fertility and fecundity. No changes were noted in sexual behaviors in the female rats or in female hamster pups exposed at the end of organogenesis [33]. Lack of vaginal opening and reduced fertility were also noted in prenatally exposed hamster females.

Similar effects to those observed with TCDD have been noted in studies using the dioxin-like PCB congener #169 [34,35], suggesting that the Ah receptor is involved in the reproductive effects observed. Premature reproductive aging has previously been reported for a commercial mixture of PCBs, Aroclor 1221, which contains few dioxin-like congeners [36]. This mixture also exhibited estrogenic activity, while higher chlorinated mixtures, such as Aroclor 1242, 1254, and 1260, did not. Prenatal exposure in mice to the dioxin-like PCB #77 has recently been shown to reduce the number of germ cells in the ovaries [37], which could potentially lead to premature reproductive aging. Sager and Girard [38] have also reported that perinatal exposure of rats to Aroclor 1254, the mixture that contains the highest concentration of dioxin-like PCBs, led to delayed puberty and decreased fertility in female offspring. Male pups exhibited decreases in accessory sex organ weights, decreased fertility, and increased testes weight [39]. No effects were seen on testosterone levels. Lundkvist [40] observed similar effects of delayed puberty and decreased sex gland weights in PCB-exposed guinea pigs. Overall, the data suggest that many of the effects of PCBs appear similar to those reported for dioxin, suggesting that these effects are mediated via the Ah receptor.

4. Hormonal effects of PHAHs during development

None of the dioxin-like developmental effects appear to be clearly estrogenic or anti-estrogenic. However, a number of the effects recently reported to be associated with exposure to both dioxin and nondioxin-like PHAHs may involve alterations in thyroid hormone levels. Cooke and coworkers [41] have recently shown that transient neonatal hypothyroidism is associated with enlarged testes. In agreement with the earlier study from Sager [39], these investigators [42] have found that prenatal exposure to PCBs also caused enlarged testes and increased testicular sperm count, associated with effects on Sertoli cells. In contrast, as reviewed above, dioxin causes a permanent decrease in epididymal and

ejaculated sperm counts, with little effect on testicular sperm count [30].

Dioxin and dioxin-like PCBs, #77 and 126, cause slight decreases in T_4 levels in weanling rat pups, with no effects on the dams following exposure on gestation days 10–16 [43]. In contrast, much larger effects on circulating T_4 levels were noted in pups exposed prenatally to PCB #118, a congener with limited dioxin-like activity, and PCB #153, a di-ortho substituted PCB with extremely limited ability to bind to the Ah receptor [44]. No effect was seen with the lower chlorinated PCB #28. In addition to changes in circulating thyroxin levels, PCB #118 exposure led to histological changes in the thyroid gland suggestive of elevated TSH levels. Morse et al. [11] have suggested that the PCB-induced decreases in plasma T_4 are due in part to induction of UDP-glucuronyl transferase. The decrease in circulating T_4 appears to be associated with an increase in Type II deiodinase, which converts T_4 to T_3. This increase in a brain-specific form of deioidinase activity may be a result of transient hypothyroidism in the developing brain. Could this play a role in the reported neurotoxicity of PCBs? In fact, Ness et al. [44] have suggested that prenatal hypothyroidism would be consistent with the observed neurobehavioral effects such as spatial learning deficits and altered motor activity. The critical role of thyroid hormones in brain development has been supported by recent studies by de Ku et al. [45] who demonstrated that thyroxine supplementation was able to reverse the decline in choline acetyltransferase activity in hippocampus and basal forebrain of neonatal rats induced by Aroclor 1254, a highly chlorinated PCB mixture containing significant amounts of dioxin-like PCBs. The potential ability of supplemental thyroxine to reverse PCB-induced neurological effects has also been recently demonstrated by Goldey et al. [46]. These investigators have shown that Aroclor 1254 exposure during development reduces circulating thyroid hormone concentrations and causes hearing deficits in rats [47] which are similar to those caused by the potent thyroid antagonist, PTU.

Effects reported on the cholinergic system may be due to the dioxin-like PCBs since neonatal exposure to PCB #77 has been reported to alter muscarinic cholinergic receptors and spontaneous motor behavior in mice [48]. Exposure of developing rats every other day from gestation days 10–20 to PCB #118 or 126 resulted in poorer visual discrimination and higher activity [49]. The potent dioxin agonist, #126, was more effective than #118. These neurobehavioral effects in the offspring occurred in the absence of clinical maternal effects or fetotoxicity. Decreased visual discrimination [50] as well as a decrease in active avoidance learning was also seen following prenatal exposure in rats to the chlorinated PCB mixture, Clophen A30, which contains primarily non-dioxin-like PCBs. Lactational exposure had no effect on these parameters. Of interest are the permanent neurobehavioral effects observed in monkeys exposed both prenatally and lactationally to both Aroclor 1016 and Aroclor 1248 [51]. The deficits observed with the more highly chlorinated mixture on delayed spatial alternation were quite dramatic. Both mixtures caused effects on discrimination reversal learning. The PCB levels in the mother's milk were within the range observed in some human populations. The possibility that neurobehavioral effects of PCBs are, at least in part, associated with the dioxin-like congeners is strengthened by the demonstrated changes in locomotor activity and rearing behavior in rats exposed perinatally to TCDD [52]. In contrast, no effects were observed in prenatally and lactationally exposed male rats in regards to sexually dimorphic behaviors [30].

Neurobehavioral effects of PCBs have been reported in both animals and humans [53]. Whether the observed effects are due to the dioxin-like congeners, the non-dioxin-like congeners, or to the combination is unclear. Children exposed prenatally to heat-degraded PCB mixtures in Japan ('Yusho') and Taiwan ('Yu-cheng') have multiple problems including developmental delays, IQ deficits, ectodermal dysplasia, and growth retardation [54]. Problems at puberty have also been noted in the young men [55]. Behavioral deficits have been noted in children whose mothers had slightly elevated levels of PCBs (reviewed in [53]). Recent studies from the highly exposed Yu-Cheng cohort have noted PCB-induced alterations in auditory event-related P_{300} potentials, suggesting an alteration in cognitive function [56]. Decreased neuro-op-

timality and hypotonicity correlated with the dioxin-like PCBs in infants from within the general population in the Netherlands [57]. Whether this is related to the dioxin-like PCB-associated decrease in circulating T_4 levels in this background population remains to be determined [58].

While it is clear that exposure to high levels of PCBs is associated with clearly adverse effects in the developing offspring, the mechanism of these effects is not clear. The recent observations that differential responses can be measured within the background population, when the population is stratified according to their dioxin-like equivalencies, suggest that subtle effects may be occurring at relatively low levels. The mechanism of such responses remains unknown. However, there is no evidence of enhanced fetal death in populations which may have elevated PCB levels due to increased consumption of PCB-contaminated sport fish [59]. Paternal exposure to TCDD appears to have no effect on pregnancy outcome [60]. Of course, the lack of adverse effects in these two studies is entirely predictable based on animal data.

5. Conclusions

An important message from epidemiological studies is that adverse effects of dioxin can be detected in highly exposed populations, such as those resulting from the rice oil poisonings in the Far East. Recent studies suggest that subclinical effects may also be present within the background population. What does this suggest for future investigations? Firstly, it may not be sufficient to look only for overt alterations in an individual. Instead, the study may need to focus on the distribution of the population. This is a situation reminiscent of that observed with lead. Subtle effects of hormonal alterations during development may put the population into an 'at risk' category, which may only be revealed under stressful conditions or by insightful measurements.

All of the developmental effects discussed above may involve multiple mechanisms. While the dioxin effects, and those of the dioxin-like PCBs, clearly require the Ah receptor, it is essential to understand that the function of this receptor system is similar to that of any hormonal system and involves complex combinatorial interactions. Dioxins initiate a cascade of biochemical changes resulting in alterations in growth and differentiation. How intricate physiological networks and signalling pathways are perturbed by the non-dioxin-like PCBs remains to be determined.

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Dose-response relationships for carcinogens

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Abstract

Biotransformation of chemical carcinogens involves both metabolic activation and detoxication. The molecular dose present on DNA as adducts represents a balance between these two pathways (formation) and DNA repair. All of these are enzymatic processes subject to saturation. When none of the pathways is saturated, linear molecular dosimetry is expected, whereas if metabolic activation is saturated, a supralinear response occurs. If detoxication or DNA repair is saturated, a sublinear response occurs. With chronic exposure, steady-state concentrations of DNA adducts develop and these follow the same patterns. With several alkylating agents, multiple adducts are formed. The extent of formation is chemically defined, but different DNA repair pathways can be involved for different adducts. By understanding the molecular dose and biology of each adduct and comparing these to the dose-response for tumor induction, it may be possible to identify the most appropriate biomarkers for risk assessment. Recently, endogenous DNA adducts identical to those induced by known human carcinogens have been identified. These endogenously formed adducts may play an important role in human carcinogenesis.

Keywords: DNA adducts; Biomarkers; Carcinogens; 1,2,3-Trichloropropane; Diethylnitrosamine; Dimethylnitrosamine; Vinyl fluoride; Vinyl chloride; Dose-response

1. Introduction

When evaluating the dose-response relationship of carcinogens, it is necessary to consider absorption, distribution, metabolism, and many other factors [1,2]. External exposure to many agents whether via air, water, or food leads to internal exposure (Fig. 1). If a compound requires metabolic activation, there may be a saturable pathway that will be less effective at high versus low doses. There are also detoxication pathways and saturation of these enhances the toxicity at high doses. Ultimately the balance between activation and detoxication determines the amount of the ultimate carcinogen available to cause heritable damage. For most agents, binding to DNA is the prime target, but binding might also occur with RNA or to protein. DNA adducts are subject to repair, whereas protein and RNA adducts are not. When DNA repair is saturated, greater numbers of DNA adducts accumulate per unit of exposure. The end result of this scheme is that the biologically effective dose of a chemical can have a complex dose-response relationship. There may also be differences in the efficiency of different DNA adducts to cause mutations; however, none will cause mutations in the absence of cell proliferation. If mutations occur at a critical site in the genome relative to carcinogenesis, they may provide the

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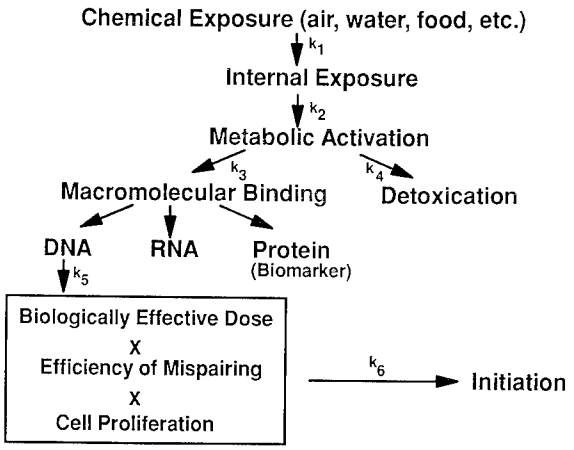


Fig. 1. Following exposure, a chemical undergoes distribution to various tissues and may be metabolically activated and/or detoxified. The balance of this product is available to covalently bind to macromolecules. Binding to DNA can be further modified by DNA repair, yielding the biologically effective dose of each adduct. K_2, K_4, and K_5 are saturable processes that can be different for high versus low doses. If DNA adducts are present when a cell divides, they can lead to mutations involved in initiation and progression of cancer.

initial step of initiation, or the genetic lesions involved in progression of neoplasia.

The different shapes of simplified dose-response curves are shown in Fig. 2. If nothing is saturated the response is linear (Fig. 2, curve *a*). Saturation of metabolic activation results in a supra-linear dose-response curve (Fig. 2, curve *b*). In this instance, the increase in toxic effect

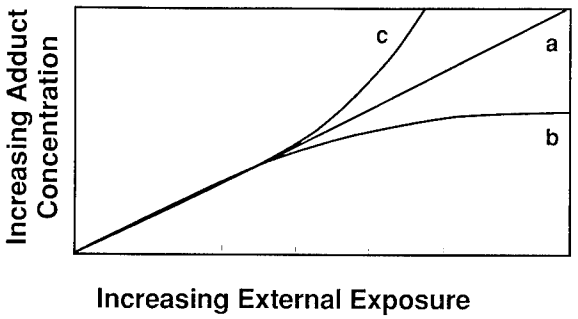


Fig. 2. Theoretical dose-response curves. Curve *a* is linear and is expected whenever none of the processes in Fig. 1 are saturated. It is also the default assumption in risk assessment. Curve *b* is supralinear and is expected whenever metabolic activation is saturated. Curve *c* is sublinear and is expected when detoxication or DNA repair is saturated. Tumor response curves are further modified by exposure-related cell proliferation.

(i.e. adducts per unit dose) is much less at higher external doses. On the other hand, saturation of detoxication or DNA repair results in a sublinear dose-response curve (Fig. 2, curve *c*). In this instance, at higher external doses there is a greater amount of damage per unit dose than at low doses. This perspective is very important in understanding the dose-response for tumor incidence or other toxic events being induced at the maximum tolerated dose and in extrapolating to exposures that may be orders of magnitude lower.

2. Examples of molecular dosimetry

One example illustrating this point is the shape of the dose-response curve for O^6-methylguanine (O^6MG) following single doses of dimethylnitrosamine covering five orders of magnitude [3]. The response with N7-methylguanine (7MG), the major DNA adduct, is linear over the entire dose range. For O^6MG, the theoretical amount formed is 1/10th that of 7MG. At lower doses the amount of O^6MG present is about 1/100th the amount of 7MG, reflecting the role of DNA repair. At higher doses the removal process is saturated and the slope of the dose-response for this adduct increases. Since O^6MG is the adduct causing most of the mutations with this compound, this saturation of DNA repair plays an important role in understanding the induction of mutations and cancer.

Another well studied example is diethylnitrosamine (DEN). DNA adducts are much like other pharmacokinetic entities (e.g. metabolites) in that on chronic administration, they attain steady state concentrations. The steady state concentration reflects that with chronic exposure, the amount being formed each day equals the amount being repaired or lost that day. By plotting steady state data it can be determined if a linear or non-linear dose-response curve is present. For DEN, the data for 4 weeks of exposure shows a linear dose-response curve for O^4-ethylthymidine (O^4ET) from 0.4 to 40 ppm [4]. At low doses, there is a 1:1 relationship between O^4ET and O^2-ethylthymidine (O^2ET), another promutagenic adduct. However, at high doses, repair of O^2ET adducts is saturated and

the curve is non-linear, with O^2ET increasing nine times faster than O^4ET [5]. O^4ET results in transition mutations at thymines, while O^2ET causes transversion mutations. Therefore, the type of mutations induced will be different at high doses than at low doses, and this is likely to be important in carcinogenesis.

(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1- butanone (NNK) is a tobacco specific nitrosamine causing tumors in the nasal cavity, lung, and liver in rats [6]. No dose-response relationship was evident for lung tumors between 5 and 50 mg/kg NNK. Belinsky et al. [7] have shown that in rat lung, O^6MG exhibits a supra-linear dose-response curve that is representative of saturation of metabolic activation. Using high dose data from such a bioassay would underestimate the risk associated with exposure to lower doses. However, it is even more complicated. Dark field microscopy of radio-labeled NNK in rats revealed much greater deposition of NNK in cells lining the small airways. This radioactivity was shown to be localized in the Clara cells, which had very high adduct levels. When the dose-response relationship for lung tumors was compared with the dose-response relationship for O^6MG, the molecular dose revealed a linear response.

Vinyl chloride (VC) is a known human carcinogen and VC and vinyl fluoride (VF) induce hemangiosarcomas in rats and mice [8]. We have recently used molecular dosimetry of their DNA adducts to better understand carcinogenesis by these chemicals [9]. A clear supralinear exposure-relationship was shown for the promutagenic DNA adduct, N^2,3-ethenoguanine (EG), with saturation of metabolic activation at 250 and 2500 ppm VF (Fig. 3). Of great interest was the clear presence of endogenously formed EG in both rats and mice using GC/high resolution MS. Endogenously formed EG was also detected in 10/10 human liver samples (Fig. 4), with a mean value of 3 per 10^7 guanines and a range from 0.7 to 7. To put this in perspective, this would be the amount of EG formed by exposures of 2–25 ppm VF or VC using the linear portion of the exposure-response shown in Fig. 3. Presently, the source of endogenous EG is unknown.

The molecular dose of ethylene oxide has also been characterized using GC/high resolution MS and this methodology has been applied to understanding the metabolism of ethylene [10]. Again, endogenous formation of 7-hydroxyethylguanine (HEG) was clearly demonstrated in control mice, rats and unexposed humans (Table 1). Exposure to ethylene oxide caused a linear increase in HEG at 3 and 10 ppm. Ethylene caused a supralinear exposure-response, with saturation of metabolic activation at exposures of 1000 ppm and higher. When the relationship between DNA and hemoglobin adducts was examined following 4 weeks of exposure, the slope of the ethylene plot was greater with data from liver and lung, i.e. there were more DNA adducts per hemoglobin adduct, suggesting that these tissues were activating ethylene to ethylene oxide.

One of the most nonlinear dose-responses for cancer induction in rats is that of formaldehyde. The dose-response curve for the induction of squamous cell carcinoma in the nose of rats exhibited no increase in tumors at 2 ppm, a 1% incidence at 5.6 ppm, and a 50% incidence at 15 ppm [11]. Thus, a 2.5-fold increase in dose resulted in a 50-fold increase in tumors. Formaldehyde undergoes detoxication via a glutathione-mediated pathway and only the non-detoxified material is available to bind to DNA and form DNA-protein cross links. Heck and Casanova [12], examined the dose-response of covalently bound formaldehyde per ppm (i.e. adducts divided by dose), and demonstrated that there were 4- to 7-fold less DNA-protein cross links at low doses than at high doses because of saturation of the detoxication pathway. Furthermore, Monticello et al. [13] repeated the formaldehyde bioassay using a mechanistically designed study which included an additional exposure group of 10 ppm. They confirmed that the slope for nasal cancer changed at exposures above 6 ppm, whereas the molecular dose was similar per unit of exposure, but 4–7 times greater than that observed at 2 ppm and lower. No increase in cell proliferation was induced by exposures of 6 ppm or less, while a marked increase in the slope for cell proliferation occurred at 10 and 15 ppm across different time points ranging from 3–18 months. Therefore,

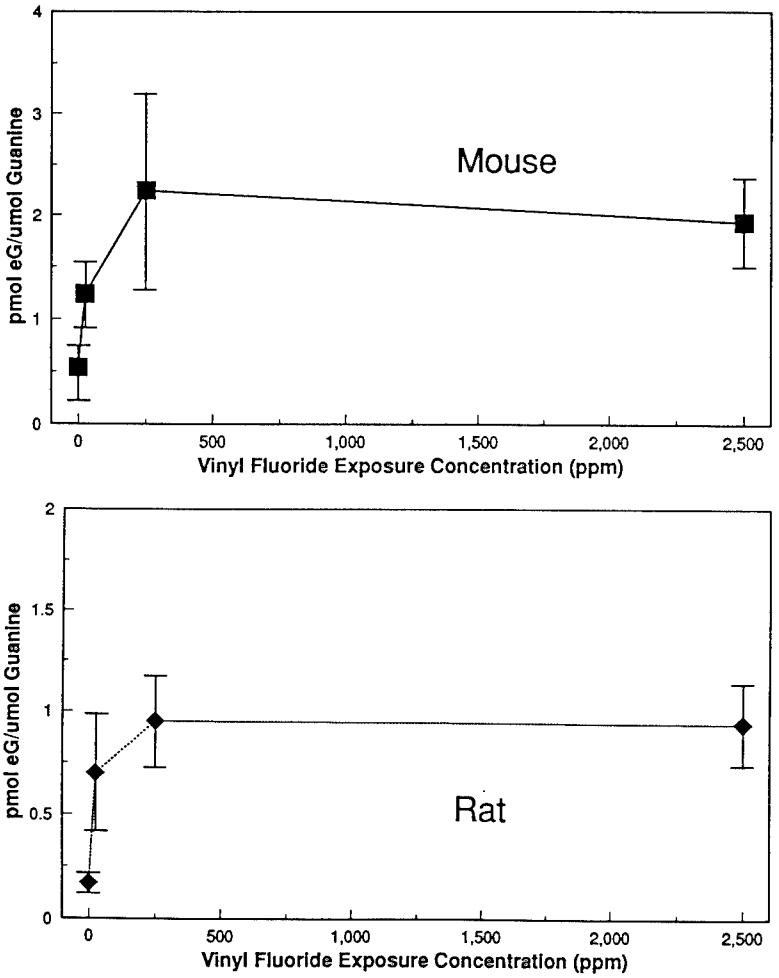


Fig. 3. Steady-state exposure response curves for N^2,3-ethenoguanine in liver DNA of rats and mice exposed to vinyl fluoride for 12 months. Note the supralinear exposure-response due to saturation of metabolic activation.

between 6 ppm and 15 ppm, the molecular dose was linear, but cell proliferation was non-linear and identical to tumor incidence. It is very difficult to argue against this not being the driving mechanism for the great increase in tumors at 10 ppm and 15 ppm.

Molecular dosimetry can be used in risk assessment in several different ways. First, as shown above, it can be used for high to low dose extrapolation to identify saturation of metabolic activation, detoxication, or DNA repair, and to extend the observable range of data. Second, molecular dosimetry can be used to improve route to route extrapolation. For example, a gavage bioassay can be extrapolated to exposure by inhalation or drinking water if these are more relevant for human exposure. This has recently been done for 1,2,3-trichloropropane [14], where it was found that exposure via gavage resulted in approximately two times more DNA adducts than exposure via drinking water (Table 2). In addition, gavage exposure caused a significant increase in cell proliferation, while drinking water did not. This strongly suggests that gavage exposure will overestimate the carcinogenic potency of this chemical.

A third area for molecular dosimetry to contribute is in assessing species differences in metabolism. This approach is being applied to butadiene to improve the accuracy of human risk

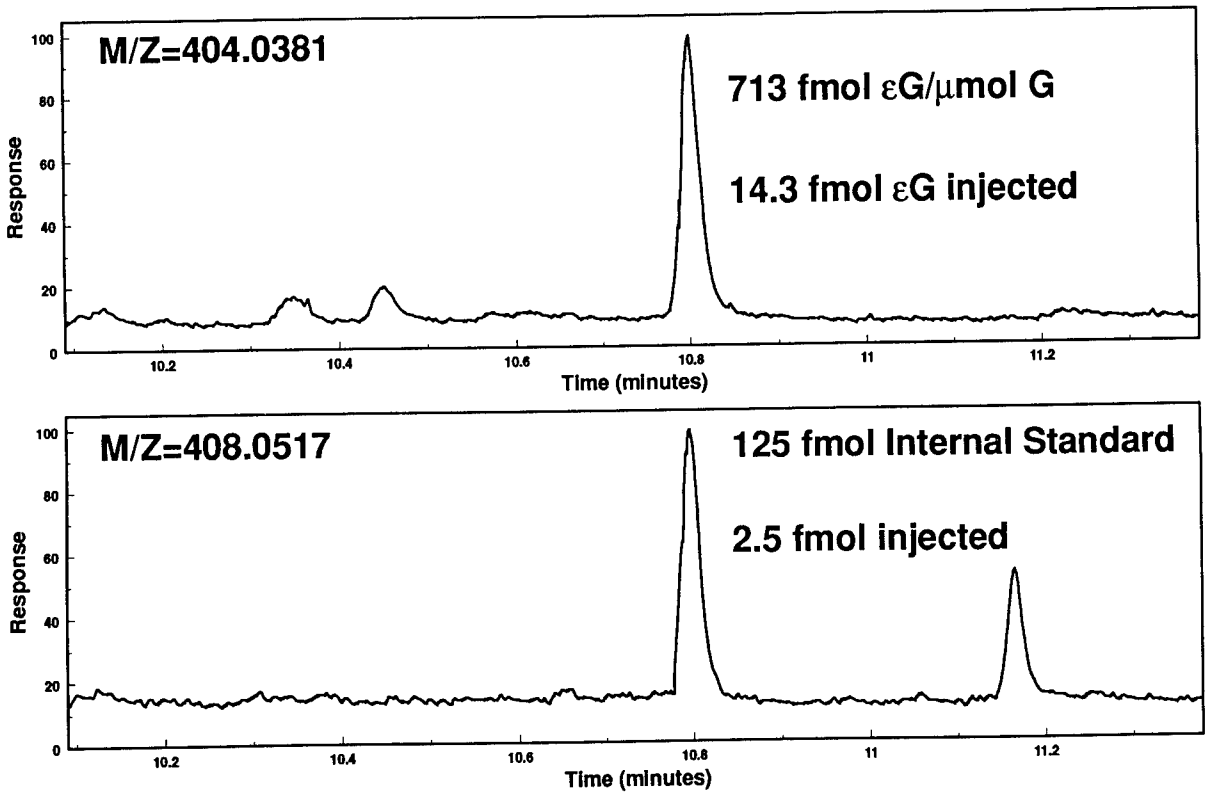


Fig. 4. GC/High resolution mass spectra of liver DNA from a 56-year-old man that had no known exposure to vinyl chloride showing the presence of endogenously formed N^2,3-ethenoguanine.

Table 1
7-Hydroxyethylguanine concentrations in liver DNA in unexposed humans and rats and in rats exposed to ethylene or low doses of ethylene oxide

Species	Exposure	HEG (pmol/μmol guanine)
Human	Unexposed	3.0 ± 1.0
Rat	Unexposed	0.2 ± 0.05
Rat	40 ppm Ethylene	1.05 ± 0.25
Rat	1000 ppm Ethylene	4.31 ± 0.25
Rat	3000 ppm Ethylene	6.49 ± 1.97
Rat	3 ppm Ethylene oxide	1.78 ± 0.87
Rat	10 ppm Ethylene oxide	3.60 ± 0.82

Table 2
Effect of exposure route on DNA adduct formation (pmol/μmol guanine) by 1,2,3-trichloropropane

Organ	Drinking water	Gavage
Forestomach	86.8 ± 73.2	123.1 ± 10.3
Glandular stomach	43.2 ± 5.9	42.5 ± 4.6
Kidney	81.9 ± 41.5	193.1 ± 64.4
Liver	185.5 ± 83.9	374.9 ± 109.2

assessment. While quantitative data are not yet available, the approach may be of interest and is presented below. GC/high resolution MS methods are being developed for the DNA and hemoglobin adducts of butadiene's mono- and diepoxide. The absolute and relative amounts of each DNA adduct will be determined in rats and mice, and the absolute and relative amounts of the hemoglobin adducts will be determined in

rats, mice and humans. This will demonstrate the preferred metabolic pathways for all three species to help select the most appropriate species for assessing human risk and will provide data on the range of interindividual variability of metabolism in humans.

3. Conclusions

We are beginning to understand mechanisms of human and animal carcinogenesis at a level that can improve the accuracy of human risk assessment [15]. We are starting to understand some of the genes responsible for cancer in humans and animals such that laboratory animal studies and epidemiology studies are beginning to include such components as genetic susceptibility factors, molecular dosimetry, and mutational spectra in oncogenes, tumor suppressor genes and other surrogate genes. These in turn will feed into the risk assessment paradigm. We will also better understand some of the interindividual differences that lead to predisposition to cancer. Many of these predispositions can be expected to be more variable in human populations than they are in inbred laboratory animals. Presently, some of the conservatism that goes into risk assessment is based on such assumptions. It is likely that we will be able make real determinations on such issues in the near future, rather than relying on defaults and uncertainty factors in risk assessment.

Acknowledgements

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Monitoring of human exposure to carcinogens through DNA and protein adduct determination

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Abstract

For genotoxic carcinogens, exposure assessment may be achieved by measurements of the extent of covalent interaction (adduct formation) that has occurred between the carcinogen and macromolecules such as DNA, haemoglobin and albumin. Adducts for many carcinogens have been found in supposedly unexposed populations. This signifies either that endogenous processes contribute to this DNA/protein modification, or that there are exogenous exposures to these carcinogens that were not previously recognised. Notable examples where 'background' genotoxic modification has been found include damage caused by low molecular weight alkylating agents and hydroxyl radicals. The significance of the existence of these adducts to genotoxic risk is as yet unknown.

Keywords: DNA adduct; Protein adduct; Carcinogens; Biomonitoring

1. Introduction

It is over 20 years since the concept was originated of biomonitoring carcinogen exposure through measurement of the interaction products (adducts) formed by carcinogens with nucleic acids and proteins. To date, only three macromolecules have received extensive study of their adduct formation: DNA, haemoglobin and albumin. DNA was an obvious selection based upon the belief that it was a 'target molecule', and that the extent of adduct formation there would bear some relationship with subsequent stages in the carcinogenesis process (e.g. mutation). In practice, the DNA sampled for human biomonitoring is normally from a non-target site for carcinogenesis, (where the spectrum of adduct formation may possibly not reflect that in the target organ). Haemoglobin and albumin are two further non-target site macromolecules that have been used for biomonitoring, whose choice was governed largely by their availability and long lifetime. Adducts on these proteins are not subject to enzymic repair processes and, if the adducts are chemically stable, their measurement may be used as a monitor of exposure to carcinogens over the lifetime of the proteins.

The relationship between adducts measured on proteins and adducts on DNA (non-target and target site) is variable according to the carcinogen, exposure conditions, etc. For many compounds, there appears to be a proportionality between protein adducts and DNA adducts, especially at low doses of carcinogen [1]. The proportionality factor, however, will vary

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according to the exposure and individual studied. In typical cases of human exposures to carcinogens, there are low doses to a multitude of compounds under exposure conditions that are unknown (e.g. exposure to environmental pollution), and it may therefore not be possible to predict, accurately, DNA adduct formation based upon a measurement of a protein adduct.

Despite these problems, measurement of non-target protein and DNA adducts has been demonstrated to be an effective means of monitoring exposure to genotoxic compounds. If (a) the adduct measured is quantitatively related to the adduct in the DNA in the target cell, and (b) the adduct in the DNA target cell is quantitatively associated with development of mutation and subsequent stages of the carcinogenesis process, then measurement of non-target adducts may also have value as a carcinogenic risk monitor. With the limited data so far available, the associations (a) and (b) above appear to be variable according to the compound studied.

The analytical methods that have been most widely used for detecting adducts include:

Protein adducts: gas chromatography-mass spectrometry, immunoassay, HPLC with fluorescence detection;

DNA adducts: ^{32}P-postlabelling, immunoassay, HPLC with fluorescence or electrochemical detection, mass spectrometry, atomic absorption spectrometry, competitive repair assay, accelerator mass spectrometry, (only for ^{14}C-labelled carcinogens).

The application of these methods for monitoring exposure to carcinogens from occupational, dietary, medicinal, and environmental (e.g. urban pollution and cigarette smoking) sources has been extensive (cf. review by Farmer [2]). A significant discovery has been the fact that adducts for many carcinogens have been found in supposedly unexposed populations. These are particularly apparent for low molecular weight alkyl adducts and for hydroxyl radical damage. The origins of this 'background' damage and its genotoxic significance are unknown. Below are summarised some representative examples of adduct measurement carried out in our laboratory, where background levels of adducts have been detected.

2. Covalent adduct formation in haemoglobin

The amino acids in haemoglobin that react with genotoxic carcinogens are those with nucleophilic side chains (e.g. cysteine, histidine, aspartic acid, glutamic acid, lysine) and the N-terminal amino acid, which is valine in both the α- and the β-chain of human globin. Analytical methods for adducts at all of these sites have been developed [3]. Thus for cysteine, the adducts that have been detected include those from aromatic amines, styrene oxide, benzene, acrylamide, acrylonitrile, and methylating/ethylating agents; for histidine, low molecular weight epoxides; for carboxylic acids (aspartic, glutamic, C-terminal amino acid), tobacco specific nitrosamines, polycyclic aromatic hydrocarbon epoxides, and styrene oxide; and for N-terminal valine, epoxides (e.g. of ethylene, propylene, butadiene, styrene), acrylonitrile, and acrylamide.

Particular attention has been placed in our laboratories on the analysis of N-terminal valine adducts. The method for this involves a modified Edman degradation procedure, originally developed by Törnqvist et al. [4]. This procedure cleaves the adducted N-terminal valine from the protein chain, liberating a thiohydantoin that may be analysed by GC-MS. For example, exposure to ethylene oxide (and other hydroxyethylating agents) may be monitored by determining the thiohydantoin generated from the adduct N-(2-hydroxyethyl)valine (HOEtVal). Using this method, we have shown that occupational exposure to ethylene oxide yields up to 13 nmol HOEtVal/g globin and that smoking 10 cigarettes/day gives ca. 70 pmol HOEtVal/g globin [2,3]. Of particular interest was the fact that supposedly unexposed individuals contained ca. 50 pmol HOEtVal/g globin [5]. The source of this is believed to be partially from endogenous ethylene, although a recent study of ours has shown that exhaust fumes contribute towards human HOEtVal levels (Autrup and Farmer, unpublished data). We have also demonstrated that new-born babies possess a background level of HOEtVal and that the level of adducts is much enhanced in the babies of smoking mothers [6]. HOEtVal levels of the babies were linearly

Table 1
Background levels of adducted amino acids in protein

Amino acid	Adduct	Level	Reference
Valine	2-(Hydroxyethyl)	31 pmol/g globin	[7]
	Methyl	500 pmol/g globin	[8]
	2-Cyanoethyl	13 pmol/g globin	[9]
Cysteine	Methyl	16.4 nmol/g globin	[8]
	2-Carboxyethyl	8.5 nmol/g globin[a]	[8]
	4-Aminobiphenyl	0.17 pmol/g Hb	[8]
Histidine	2-(Hydroxyethyl)	1.6 nmol/g globin	[8]
Carboxylic acids	Benzo[*a*]pyrene	700 pmol/g albumin	[10]

[a] After acidic hydrolysis.

correlated with the levels of the mothers. Ongoing work has confirmed these data, comparing mothers' and umbilical cord haemoglobin, in a larger population of Danish women (Autrup and Farmer, unpublished data).

In a recent international study funded by the European Union, we were given the opportunity to compare HOEtVal levels in the globin of several European populations living in areas of varying environmental pollution. In this study, a rural 'control' population from Yorkshire, UK had significantly more ($P < 0.01$) HOEtVal than populations living in more polluted areas in Swansea, UK and Copenhagen, Denmark (35.3 ± 7.6 compared to 27.1 ± 6.3 and 20.9 ± 9.1 pmol HOEtVal/g globin, respectively [7]). There appear to be further unknown environmental parameters governing the amount of HOEtVal production. HOEtVal is just one example of many adducts where background levels have been demonstrated (Table 1). The existence of these adducts presumably reflects exposure to alkylating agents, and is commonly matched by the existence of 'background' DNA adducts containing the same alkyl groups (see below).

3. Covalent adduct formation in DNA

The most general approach for detecting DNA adducts is the ^{32}P-postlabelling assay. Originally developed by Randerath et al. [11], this assay is capable of screening for adducts from most genotoxic carcinogens at sensitivities that may reach as low as 1 adduct/10^{10} nucleotides. Although a highly sensitive method, it does not give direct information regarding the chemical nature of the adduct. Its use has been extensive (for applications, see review of Beach and Gupta [12]) with especial application in human samples for the detection of bulky (e.g. polycyclic aromatic hydrocarbon) adducts. Measurable background levels have been detected in 'control' human DNA samples. The source of these is unknown, although environmental factors clearly play a role, e.g. the amount of aromatic DNA adducts in lymphocytes measured by postlabelling has been shown to be increased in winter compared to summer, presumably because of the greater burning of fossilised fuel in the winter months [13].

We have recently demonstrated the presence of background levels of adducts in a truly rural population living in the Shetland Isles (Cole et al., unpublished data). In January, 1993, an oil tanker *Braer* ran aground on the southern tip of the Shetland Isles and there was extensive local pollution with oil. Samples of blood were collected from the local, potentially exposed population and from an area well removed from the disaster. Immediately after the accident, the 'exposed' group had DNA adduct levels (measured as the 'diagonal radioactive zone' in the postlabelling procedure) at a mean level of 6.6 adducts/10^7 nucleotides. The control group had a level of 5.6 adducts/10^7 nucleotides, which was insignificantly different. There was thus no evidence of genetic damage caused by the spill.

Levels of background DNA adducts have recently been measured in non-smoking populations in several other European locations (Farmer et al., CEC STEP programme, unpublished data). Preliminary indications are that

levels in populations in urban locations (e.g. Copenhagen 8.6 adducts/10^7 nucleotides, Athens 9.4 adducts/10^7 nucleotides) are higher than those in rural surroundings (Denmark 4.3 adducts/10^7 nucleotides, Crete 5.6 adducts/10^7 nucleotides).

Detection procedures of more chemical or biochemical specificity, such as HPLC-ECD, fluorescence, GC-MS, and immunoassay, have been used to quantitate specific adducts in DNA. Low molecular weight adducts (e.g. N-7-methylguanine [14], N-7-(2-hydroxyethyl)guanine [15], N^2,3-ethenoguanine [16], the malondialdehyde adduct of guanine [17]), are present in control DNA, indicating either exogenous or endogenous exposure to low molecular weight electrophilic agents.

DNA also appears to be constantly exposed to oxygen radicals, e.g. the hydroxyl radical HO$^\bullet$. Thus, for example, one of the products of hydroxyl radical attack on DNA, 8-hydroxyguanine, has been detected by HPLC/ECD in a range of animal and human tissue DNA at a range of 1–30 adducts/10^6 DNA bases [18]. Thymine glycol, which is another product of oxidative DNA damage, has been detected in human urine at a concentration of 0.39 ± 0.36 nmol/kg/day [19]. This urinary modified base presumably results from repair of oxidatively modified nucleic acids.

We have recently developed a GC-MS-MS procedure for the quantitation of thymine glycol in DNA [20]. This procedure involves the hydrolysis of DNA in 60% formic acid, at 140°C, derivatising the product with a t-butyldimethyl silylating agent and GC-MS selected ion recording or GC-MS-MS multiple reaction monitoring. Preliminary results indicate background levels of thymine glycol in rat DNA of ca. 3 modified bases/10^6 nucleotides and human placental DNA of 9 modified bases/10^6 nucleotides (Farooq et al., unpublished data).

A summary of some of the background levels of DNA damage that have been detected is shown in Table 2.

4. Conclusion

Satisfactory analytical techniques now exist for the measurement of many DNA and protein adducts caused by human exposure to genotoxic agents. The value of these techniques as an exposure monitor has been validated, but the value as a risk monitor has not yet been general-

Table 2
Background levels of modified bases in DNA

Adduct	Tissue	Level	Reference
Guanine			
N-7-Methyl	Human lymphocytes	$2.3/10^7$	[14]
N-7-(2-Hydroxyethyl)	Rat liver	$2.5/10^6$	[15]
N^2,3-Etheno	Human liver	$1.7/10^7$	[16]
Malondialdehyde	Human liver	$9/10^7$	[17]
8-Hydroxy	Human leukocytes	$1.2/10^6$	[21]
Adenine			
1,N^6-Etheno	Human liver	$0–14/10^9$	[22]
Cytosine			
3,N^4-Etheno	Human liver	$0–26/10^9$	[22]
Thymine			
5,6-Glycol	Human placenta	$9/10^6$	Farooq et al., unpublished data
O^4-Ethyl	Lung (ex-smokers)	$1–7/10^8$	[23]
All bases			
Bulky (PAH) adducts	Human lymphocytes	$1–5/10^8$	[13]

ly demonstrated. (The exception to the latter statement is the work of Qian et al. [24], where adducts of guanine with aflatoxin B_1 in urine were statistically correlated with liver tumour formation. This is a clear demonstration of the potential of adduct measurement as a risk monitor.)

The existence of background levels of adducts presumably reflects the existence of a 'background' genetic risk. Human DNA appears to be constantly alkylated and oxidatively damaged, with simultaneous continuous repair of the modified bases. The result is a plateau low level of damage in 'control' DNA, whose magnitude is dependent on the efficacy of DNA repair processes. For 8-hydroxyguanine in DNA, the background levels increase with age in the rat, as DNA repair mechanisms presumably become less efficient. It is not possible, as yet, to indicate the size of the genetic hazard caused by these background levels of DNA damage. Each adduct will have a different mutagenic effectiveness, and promotional influences may be instrumental in governing whether or not the lesion is detrimental to the cell. However, it would be reasonable to assume that populations with a higher level of adducts would, as a group, be at a higher risk of acquiring cancer.

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Molecular epidemiology and human risk monitoring

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Abstract

Molecular epidemiologic research involves the identification of relations between previous exposure to some putative causative agent and subsequent biological effects in a cluster of individuals in populations. There is intensive current research in the field of molecular epidemiology, and this research has a direct impact on risk assessment processes. Many of the challenges facing risk assessors today can be addressed by research focused on developing a better understanding of (a) exposure characteristics or assessment, (b) the relationship between exposure and dose, and (c) the ultimate exposure/dose effect response relationship. Results from this research can be used to design and implement preventive interventions in at risk populations. Thus, the application of research in exposure assessment and molecular epidemiology to risk assessment and preventive interventions makes this a core program for public health.

Keywords: Molecular epidemiology; Molecular biomarkers; Carcinogenesis; Exposure assessment

1. Introduction

Epidemiological research involves the identification of relationships between previous exposure to some putative causative agent and subsequent biological effects in a cluster of individuals in populations. These relationships are frequently difficult to fully characterize because of difficulties in accuracy quantifying exposure, dose and effect. Exposure is a relationship between a chemical, physical, or biological agent and an individual or group of people that provides an opportunity for delivery of the agent from external environment to internal body. Dose is the amount of agent actually deposited within the body. Typically, the distinction between exposure and dose is blurred, although in reality the same exposure may lead to significant differences in dose. Effect is the biological response to the agent. Methods to more accurately and sensitively characterize exposure, dose and effects are particularly needed in research involving environmentally occurring toxic agents.

Molecular epidemiology focuses on the use of biomarkers in epidemiologic research. Molecular biomarkers are typically indicators of exposure, effect, or susceptibility [1]. A biomarker of exposure indicates the presence (and magnitude) of previous exposure to an environmental agent. Such a biomarker may be an exogenous sub-

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stance, an interactive product (e.g. between a xenobiotic compound and endogenous components), or other indicator. A biomarker of effect indicates the presence (and magnitude) of a biological response to exposure to an environmental agent. Such a biomarker may be an endogenous component, a measure of the functional capacity of the system, or an altered state recognized as impairment or disease. A biomarker of susceptibility indicates an unusually elevated sensitivity to the effects of an environmental agent. Such a biomarker may be the unusual presence or absence of an endogenous component, or an unusual functional response to an administered challenge. Biomarkers thus offer significant potential in clarifying the relationships between environmental agents and disease [2].

The development of putative molecular biomarkers for environmental agents should be based upon specific knowledge of metabolism, interactive product formation, and general mechanisms of action [3,4]. The validation of any biomarker-effect link requires parallel experimental and human studies. Ideally, an appropriate animal model is used to determine the associative or causal role of the marker in the disease or effect pathway and to establish relationships between dose and response. The putative marker can then be validated in pilot human studies where sensitivity, specificity, accuracy, and reliability parameters are established [5]. Data obtained in these studies can be used to assess intra- or inter-individual variability, background levels, relationship of the marker to dose or to disease status, as well as feasibility for use in larger population-based studies. It is important to establish a connection between the biological marker and exposure, effect, or susceptibility. To fully interpret the information that the marker can provide, prospective epidemiological studies may be necessary to demonstrate the role that the marker plays in the overall pathogenesis of the disease or effect. This article will summarize some of the global principles of this field and is adapted from a more detailed article that has been recently published [4].

2. Environmental exposure

Environmental agents may be broadly classified as either chemical, physical, or biological in character. A biomarker of exposure would ideally indicate the presence and magnitude of previous exposure to an environmental agent. In the absence of biomarkers, an assessment of exposure typically requires measurement of toxicant levels in the environment, and characterization of the individual's presence in, and interaction with, that environment. The measurement of toxicants in media (e.g. air, water, soil, or food) is accomplished by a wide array of analytical methodologies. Assessments of exposure include questionnaires, personal external monitors, and measurements of chemicals or physical agents in the ambient environment. Questionnaires have been extensively used to determine broad dietary exposures to compounds, smoking histories, and genetic backgrounds (in the context of susceptibility). While questionnaire data have proven useful in some circumstances (e.g. in assessing current smoking status), this approach is very imprecise for measuring other exposures, such as those occurring through diet, where the knowledge base of specific dietary chemical agents is still limited.

A complexity arising from the use of ambient measurements to determine exposure status of individuals is the heterogeneous nature of most environmental contaminations. It is rare for an agent to be evenly distributed in environmental media. One illustration of this problem is the measurement of aflatoxin levels in grains. The distribution of aflatoxins in grains is very uneven due to variable patterns of mold growth, such that the sampling procedure used to determine aflatoxins in grains often results in a greater than 100% coefficient of variation [6]. This extensive variation makes it very difficult to precisely extrapolate data on grain contamination to an individual's exposure. Given these problems, the goal for the development of specific biomarkers to assess exposure is multifold, and must include an ability to integrate multiple portals of entry, integrate fluctuating exposures over time, relate

time of exposure to dose, and examine mechanistically important biological targets. These requirements assume increased relevance when it is recognized that safety regulations designed to limit human risk are often set on the basis of ambient exposure determinations.

3. Internal dose

Given the problems described above for extrapolating ambient measurements to specific individual exposures, it has been well-recognized that measures of internal dose of a specific agent provide a clearer demonstration that a toxicant has been absorbed and possibly distributed in the body. Many direct measurements of toxic chemicals or their metabolites in body fluids and excreta (e.g. blood, urine, feces, milk, amniotic fluid, sweat, hair, nails, saliva, breath) have been done.

The measurement of the body burden of lead is an example of an agent acting as its own biomarker. It is well established that blood lead levels reflect recent environmental exposure [7]. However, some manifestations of lead toxicity, such as renal dysfunction or diminished neuropsychological performance, correlate better with other measures of body burden, such as bone or tooth levels. Furthermore, it is known that bone contains over 90% of the lead body burden [8]. Thus, the body burden of lead, as characterized by bony stores, may be the more relevant measure in relating 'dose' to effect.

In an attempt to directly measure bone lead, X-ray fluorescence has been used [9,10]. In this approach, an external radiation source is used to ionize lead atoms in bone. This ionization process leads to a rearrangement of the electrons orbiting the lead nucleus, which in turn results in emission of X-rays. The energies of these so-called fluorescent X-rays are characteristic for lead, and may be externally detected. By careful calibration of the system, the measured X-ray intensity may be converted to bone lead concentration.

4. Biologically effective dose

Internal dose measurements provide unequivocal identification of previous exposures; however, they do not provide evidence that toxicologic damage has occurred; it is this damage that possibly results in cancer or other diseases. Among the various possible biomarkers reflective of these disease endpoints, the measurement of carcinogen-DNA and protein adducts is of significant interest because they are direct products of (or surrogate markers for) damage to critical macromolecular targets. These adducts result from the covalent interaction of a chemical carcinogen (or its metabolites) with DNA or other proteins.

Many different types of analytical techniques have been devised to measure chemical-macromolecular adducts, as reviewed by Kaderlik et al. [11] and Wogan [2]. These techniques have been used to measure composite and specific DNA adducts in cellular DNA isolated from peripheral lymphocytes, bladder and colonic tissues, as well as excreted DNA adducts in the urine of humans exposed to environmental toxicants. In addition, these types of techniques have been applied in a clinical setting to examine DNA adducts of people undergoing chemotherapy with alkylating agents, in an attempt to associate adduct levels with clinical outcome [12].

In addition to monitoring carcinogen-DNA adducts in situ in DNA, the eliminated products of these adducts can be determined in urine. These urinary biomarkers have been especially amenable to comprehensive validation studies [13]. One example of these studies is the examination of the dose-dependent excretion of urinary aflatoxin biomarkers in rats following a single exposure to aflatoxin B1 (AFB1) [14]. The relationship between AFB1 dose and the excretion of the major nucleic acid adduct, AFB-N7-Gua, over the initial 24-h period following exposure demonstrated an excellent linear correspondence between oral dose and excretion of a biologically relevant metabolite in urine. In contrast, other oxidative metabolites, such as aflatoxin P1 (AFP1), revealed no linear excretion characteristics.

Similar techniques have been used for the analysis of oxidative damage products of nucleic acids excreted in urine [15]. A monoclonal antibody that recognizes 8-oxo-7,8-dihydro-2′-deoxyguanosine was isolated and used in the preparation of immunoaffinity columns to facilitate the isolation of these damage products from various biological fluids. Quantitative analysis of these adducts in urine of rats fed a nucleic acid-free diet suggests that this is the principal repair product in DNA of both eukaryotes. In addition, excretion of oxidative DNA damage products in urine has been correlated to dietary antioxidant consumption in people [16,17]. Thus, these markers may eventually be used to assess protection status as well as risk in people.

A wide variety of aromatic amines and polynuclear aromatic hydrocarbons has been found to bind at high levels to hemoglobin following environmental exposures [18]. Indeed, knowledge regarding chemical-hemoglobin adducts is much more extensive than for chemical-DNA adducts. These chemical-protein biomarkers have been particularly well characterized for the potent bladder carcinogen, 4-amino-biphenyl (4-ABP). One recent study examined the relationship between exposure to environmental tobacco smoke and levels of 4-ABP-hemoglobin adducts in nonsmoking pregnant women compared to adduct levels in those women who smoked during pregnancy [19]. A questionnaire on smoking and exposure to environmental tobacco smoke was completed by pregnant women who smoked cigarettes and those who did not smoke. Samples of maternal blood and cord blood collected during delivery were analyzed for 4-ABP-hemoglobin adducts by gas chromatography with negative ion chemical ionization mass spectrometry. The mean adduct level in smokers was approximately ninefold higher than that in nonsmokers. Among nonsmokers, the levels of 4-ABP adducts increased significantly with increasing environmental tobacco smoke level. This relationship between environmental tobacco smoke exposure and 4-ABP-hemoglobin adduct levels supports the concept that environmental tobacco smoke is a probable hazard during pregnancy.

In addition to hemoglobin adduct analyses, chemical albumin adducts have also been investigated, in particular for AFB1 exposures. In recent studies conducted in The Gambia, West Africa, a strong dose-response relationship was seen [20], similar to that previously reported in China [21]. From a practical perspective pertinent to epidemiologic studies, the measurement and quantification of the aflatoxin-serum albumin adduct offers a rapid, facile approach that can be used to screen very large numbers of people [22]. Of importance, chemical-protein markers offer the further advantage of reflecting longer exposure periods in people when compared to urinary adduct markers.

5. Relationship between exposure and disease risk

In general, the most rigorous proof of an association between exposure and disease outcome is found in prospective epidemiological studies, where healthy people are followed until the diagnosis of disease. A nested case-control study, initiated in 1986 in Shanghai, examined the relationship between markers for aflatoxin and hepatitis B virus (HBV) and the development of liver cancer [23,24]. In this study, over 18 000 urine samples were collected from healthy males between the ages of 45 and 64. In the subsequent 7 years, 50 of these individuals developed liver cancer. The urine samples for cases were age-matched and residence-matched with controls, and analyzed for both aflatoxin biomarkers and hepatitis B virus surface antigen status. A highly significant relative risk was observed for those liver cancer cases where urinary aflatoxins were detected. The relative risk for people who tested positive for the HBV surface antigen was about eight, but individuals with both urinary aflatoxins and positive HBV surface antigen status had a relative risk for developing liver cancer of about 60. These results demonstrate a relationship between the presence of carcinogen-specific biomarkers and cancer risk. Moreover, these findings provide the first demonstration of a multiplicative interaction between two major

risk factors for liver cancer, HBV and AFB1 exposure. Finally, when individual aflatoxin metabolites were stratified for liver cancer outcome, the presence of AFB-N7-Gua in urine always resulted in a two- to threefold elevation in risk of developing liver cancer. These findings extend the conclusions from rodent chemoprotection studies that monitoring urinary levels of this biomarker may be appropriate for assessing individual risk.

6. Biomarkers of effect

Biomarkers of effect represent health impairment, or an event predictive of subsequent health impairment. Health impairment includes functional changes or the occurrence of frank disease. Events predictive of subsequent health impairment include sub-cellular changes and subclinical functional changes.

A major endpoint frequently studied in environmental health research is cancer. In the context of biomarkers, it is helpful to think of clinical cancer as the final stage of a multistage carcinogenesis process [25,26]. The process starts with exposure to a putative environmental carcinogen (e.g. a biological agent like a virus, a chemical agent like benzene, or a physical agent like ionizing radiation), and progresses through initiation (involving a genetic change which results in an initiated cell), promotion (involving defects in terminal differentiation or growth control that result in a pre-neoplastic lesion), conversion (involving activation of proto-oncogenes like *ras* or inactivation of tumor suppressor genes like p53 that result in a malignant tumor), which leads to clinically manifest ('frank') cancer.

The increasing mechanistic understanding of the genetic alterations that underlie the progression from initiation to clinical cancer in the process of carcinogenesis has permitted the initial development of sensitive tests for diagnosis of oncogenes and tumor suppressor gene activities. For example, there have been many recent studies of the tumor suppressor gene, p53, the most commonly mutated gene detected in human cancers. The number and type of mutations in this gene are not equally distributed, but occur in specific hotspots that vary with tumor type [27]. Different patterns in mutation type between tumors may be consistent with different etiologies for the specific tumor types. For example, several independent studies of p53 mutations in hepatocellular carcinomas occurring in populations exposed to aflatoxin found high frequencies of guanine to thymine transversions, with clustering at codon 249 [28,29]. On the other hand, studies of p53 mutations in liver tumors from Japan and other areas where there is little exposure to aflatoxin revealed no mutations at codon 249 [30]. These data have been recently confirmed in follow-up studies in different populations [31]. In the future, it may be possible that the mutational spectrum in a target gene such as p53 can serve as a marker of exposure to, and indicate damage from, specific classes of environmental agents.

Detectable genetic changes in specific oncogenes may also prove useful as an aid in diagnosis. Recent data indicate that the specific mutations in the K-*ras* oncogene in colon tumors of an individual can be detected in the shed colonic cells found in the stool of patients with colorectal tumors [32]. In these experiments, *ras* mutations were detected in the stools of eight out of nine patients with tumors exhibiting this mutation, but were found in none of the stools of the patients whose tumor did not express this mutation. These methods may now have a direct application to the identification of people with early stage disease. A particularly compelling example is that of Hubert H. Humphrey, who died of bladder cancer in 1978. Analysis of urine samples from 1967, when he first presented with hematuria, disclosed the presence of a p53 mutation in a number of the cells present [33]. This mutation, a transversion from adenine to thymine in codon 227 of the p53 gene, was identical to that found in a fixed tissue-block specimen of Humphrey's infiltrative transitional-cell bladder carcinoma, obtained in 1976. Of major importance, no diagnosis was established in 1967, and Humphrey was followed with cystos-

copy every 6 months. A definitive diagnosis of cancer was not established until 1973, fully 6 years after p53 mutations were already present.

7. Future directions

To date, many studies of individual molecular biomarkers in experimental animals and human populations have been undertaken. These investigations show that many markers serve as indicators of exposure or dose, or as indicators of the development of altered structure, function, or disease, or as indicators of possible susceptibility to disease. The major challenge facing this field is the ability to link exposure markers to a toxicological effect. Currently, it is only through supposition that this linkage is made. Even in those cases where all of the data are consistent with a specific etiological agent and mutagenic event, such as with aflatoxin and p53 mutations, the specific information required to 'tie everything together' remains to be elucidated. Such information will eventually be gleaned from both experimental studies and molecular epidemiological investigations in human populations. Thus, the continued rapid development of the technologies used in molecular epidemiology should lead to more accurate exposure assessment as well as the development of effective intervention strategies. These marker studies will also improve and refine the ability to identify susceptible populations, thereby increasing the power of epidemiologic investigations. Finally, these tools should quickly evolve to be valuable in the policy and regulatory setting by helping to more accurately characterize the relationship between exposure and effects in people.

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The implications for risk assessment of measuring the relative contribution to exposure from occupation, environment and lifestyle: hemoglobin adducts from amino- and nitro-arenes

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Abstract

Recent progress in biomonitoring allows measurement of internal exposure of individuals ranging from occupational and life style exposures to environmental levels. Ten specific hemoglobin adducts generated by polycyclic and monocyclic nitro-arenes were measured in coke oven workers and residents living on ground contaminated with explosive wastes, respectively. Consistently, adducts were found in most 'exposed' as well as control individuals, interindividual variation being great. Adduct levels in the majority of exposed individuals were within the range of reference values (95 percentile). Although hemoglobin adduct levels do not directly reflect genotoxic potential and potency of the parent compounds, they correlate with the biologically active dose. On the basis of such target doses, the contribution of specific exposures relative to 'background' and to related chemicals can be assessed. The impact of 'relative risk' on risk perception and risk management is to provide a rationale for the application of the ALARA principle (As Low As Reasonably Achievable).

Keywords: Hemoglobin adducts; Exposure control; Risk assessment; Amino-arenes; Nitro-arenes

1. Introduction

The public is greatly concerned about exposures to genotoxic carcinogens. Since threshold levels, by definition, cannot be scientifically determined, methods for setting tolerance levels are needed, and those that are applied are strongly debated. Cancer risk can be quantified on the basis of epidemiological data only in a few cases. Therefore, results obtained from animal experiments are used to establish an exposure level associated with an incidence of one additional tumor in 10^5 or 10^6 individuals, which was decided to be tolerable. The calculation is often based on a 'unit risk', which is defined as the number of tumor cases caused by an exposure to 1 $\mu g/m^3$ in the environmental air for a life time (70 years). This concept requires a number of assumptions and transfer steps that are all associated with great uncertainties such that the figures obtained at the end are uncertain by several orders of magnitude. In addition to the open question whether animals predict human carcinogenesis, the following problems are to be considered: (1) dose-response relationships are based on external exposure, (2) high doses are mostly

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encountered and have to be extrapolated down to environmental exposures usually over several orders of magnitude, (3) species differences are simply dealt with by referring to the most sensitive species as relevant for man, (4) interindividual human variability cannot be accounted for, (5) synergistic effects with other related or unrelated risk factors are not considered. The introduction of safety or uncertainty factors certainly does not improve the scientific basis of the concept.

The target dose concept [1] leads to an alternative approach. Measuring the biologically active dose or a relevant equivalent of it in humans reduces or even avoids the problems mentioned: (1) in contrast to the external dose, not only the internal dose is measured, but also the more relevant metabolically activated part of it, (2) low dose exposures are directly determined as they occur in real life situations, (3) the data are obtained from the relevant species, i.e. man, (4) interindividual variability is observed and causes can be studied, and (5) the problem of plurifactorial exposures can be approached.

The biologically active dose of genotoxic carcinogens is best represented by the level of adducts formed between reactive metabolites and macromolecules such as DNA and proteins. Most biomonitoring procedures to date use lymphocyte DNA or hemoglobin in erythrocytes as substitutes for the true target, but it is quite conceivable – and has been demonstrated in certain cases – that adduct formation in these substitute targets correlates well with that in critical targets [2–4].

Monitoring of biochemical effect in human populations is increasingly performed, mostly in occupational settings, and has undoubtedly improved exposure control. From there to risk assessment is a big step, but the results suggest ways of assessing relative risks that may help to put risk perception on a more rationale basis and may assist the management of practical situations. Last but not least, the accumulating human biomonitoring data will eventually provide the basis for a better risk assessment.

Since our experience is with hemoglobin adducts generated by amino- and nitro-arenes, we will concentrate on these examples to develop the above points in more detail.

2. The biologically active dose of amino- and nitro-arenes

Monocyclic and polycyclic amino- and nitro-arenes occur not only in certain occupational situations, but also in food, and they seem to be ubiquitous environmental chemicals. Many of them are classified or suspected human or animal carcinogens [5]. Both types of chemicals are metabolically activated to ultimate genotoxic metabolites: the amines by N-oxidation and conjugation of the resulting hydroxylamines or hydroxamic acids [6], the nitro-arenes, among others, by reduction of the nitro-group to enter into the same metabolic pathways [7]. *N*-hydroxyamino-arenes may be considered the common precursors for the reaction with DNA leading to a promutagenic lesion, which in many cases is a C-8 adduct of guanine. Another common metabolite is the nitroso-derivative, which is formed by oxidation of the above *N*-hydroxyamino-arene and reacts with SH-groups to yield a sulfinamide. Oxidation and the reaction with SH-groups of hemoglobin occur predominantly in erythrocytes in the course of methemoglobin formation [8–10]. The hemoglobin adducts are usually stable in vivo and can readily be used as a dose monitor (Fig. 1).

For this purpose hemoglobin is precipitated from erythrocyte lysates, the sulfinamide is hydrolyzed under mild alkaline conditions, and the released amine is extracted and quantified by HPLC with UV-, electrochemical or fluorescence detectors, or, particularly with the low adduct levels present in human blood samples, after derivatization by gas chromatography-mass spectrometry (in the NCI mode). This method has several advantages. A 5- to 10-ml sample of blood is usually sufficient for analysis. The life time of erythrocytes is 120 days in humans, i.e. steady state adduct levels from exposures over the last 3–4 months are measured. The adduct forming chemical is identified by its retention time in the gas chromatogram and by its mass in

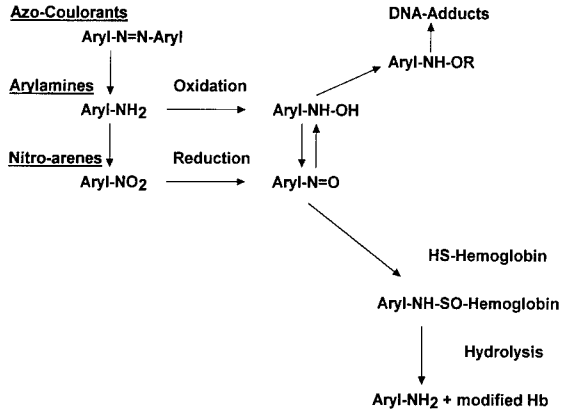


Fig. 1. Simplified scheme of the metabolic activation of amino- and nitro-arenes and the formation of DNA and hemoglobin adducts. R may be -acetyl or $-SO_3H$.

the mass spectrum. The adduct levels represent an integral measure not only over time but also from different environmental sources. Moreover, the adducts of more than one chemical can be determined. The adducts represent an equivalent for the bioavailability of critical metabolites, the *N*-hydroxyamino-arenes, and thus correlate with the biologically active dose.

A disadvantage may be that one cannot decide whether the individual was exposed to the respective amino- or the nitro-arene or both. Considering, however, that both types of arene derivatives may lead to the same critical DNA lesion, the adduct level represents a correlate for the total formation of this particular DNA lesion, which is the most relevant information for risk assessment. Another question regards the exposure to mixtures of these chemicals or the exposure to chemicals of this type in different situations. If, for instance, aniline is the cleavage product of the hemoglobin adduct, exposures may have been to nitrobenzene in the work place or from automobile exhausts, to aniline from active or passive smoking, to pesticide contamination in food, or to azo-dyes absorbed from textiles. The same sources provide a number of other related and more hazardous chemicals. They are all expected to contribute to risk. We have shown previously that DNA and protein adducts are formed by amino-arenes not only in the so called target tissue, but also in most if not all other tissues [11,12]. In at least one example, we have also shown that two amino-arenes, *trans*-4-acetylaminostilbene and 2-acetylaminofluorene, act synergistically as tumor initiators, despite the fact that they have different target tissues and form different types of DNA adducts [13,14]. We conclude from these results, that at least within these two classes of chemicals, amino- and nitro-arenes, the total exposure and the resulting total load of DNA lesions determine the number of initiated cells and thus an essential prerequiste of tumor formation. Target tissue and latency period, however, may be more severely influenced by acute and chronic toxic effects. Such effects may be produced by the chemical itself or by other risk factors.

3. Nitro-arenes as indicators for exposure to pyrolysis products

The carcinogenic properties of tars and coke oven emissions are generally attributed to the content of polycyclic aromatic hydrocarbons (PAH) [5]. Benzo[*a*]pyrene is most often used as a representative marker of exposure, and its DNA adducts in lymphocytes are used for biochemical effect monitoring. Nitro-PAH may also be present in pyrolysis products, and many of them are strong mutagens. They are also carcinogenic, but their contribution to the carcinogenic potency of such mixtures is not clear. We proposed the use of hemoglobin adducts of such nitro-PAH as exposure markers and participated in a collaborative study of coke oven workers, in which several biochemical and biological markers were analyzed [15].

We selected five representative mono-nitro-PAH for adduct analysis: 1-nitropyrene, 2-nitrofluorene, 9-nitrophenanthrene, 3-nitrofluoranthene, and 6-nitrochrysene. Coke oven workers were assigned to three different job categories: (1) working in the bottom area ($N = 18$), (2) in the middle, i.e. the bench area ($N = 71$), and (3) at the top side ($N = 12$). Controls were selected from the same area, but not associated with the company. Blood samples were processed, the

Table 1
Biomonitoring of coke oven workers hemoglobin-adducts. Percentage with detectable levels of adducts

	Exposed ($N = 102$)	Controls ($N = 19$)
1-Aminopyrene	95	89
2-Aminofluorene	91	58
9-Aminophenanthrene	89	53
3-Aminofluoranthene	56	32
6-Aminochrysene	51	21

adducts hydrolyzed and the respective amino-arenes derivatized with pentafluoropropionic acid anhydride and analysed with a Hewlett Packard 5988 GC-MS. Deuterated 9-aminoanthracene served as an internal standard. The recovery of each of the five amino-arenes was 78–89%, and the detection limit was 0.1–0.4 fg.

All five cleavage products from hemoglobin adducts were detected to various degrees in exposed and control individuals (Table 1). Surprisingly, 1-aminopyrene was found in practically all samples from exposed workers and controls, although in different amounts. Since 1-aminopyrene was the most abundant cleavage product, it will be discussed first as an exposure indicator. The box plots (Fig. 2) show on a group basis that there is exposure at the work place, and this exposure is significantly higher in the bottom area of the coke oven than in controls and those working at the bench area. Exposure of top side workers is intermediate. With regard to the risk that might be associated with the occupational exposure, however, it is much more interesting to look at the biologically active dose at the individual level (Fig. 3). Sixty percent of the blood samples from bottom workers lie within the 95 percentile of the controls, if that is taken as a reference value. Only the remaining 6–7 individuals above this cut off value may be considered at higher risk than the controls. Moreover, the adduct levels of these individuals vary considerably, and one would like to know the reasons for that. Are the high values due to excessive external exposure? This would be preventable. Are they due to life style factors? The smoking status was considered but did not correlate with adduct levels. Are they due to the individuals toxicokinetics? This could be tested by genotyping for relevant enzyme polymorphisms.

Similar results were obtained with all five nitro-PAH. Consistent adduct patterns within the groups were not seen, i.e. a job-specific pattern could not be identified. The differences between individuals, however, suggest that the metabolic capacity may indeed play a significant role. When the adduct levels from all five adducts are added,

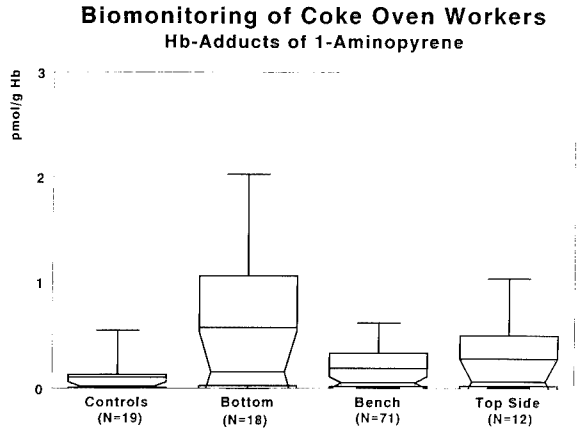


Fig. 2. Biomonitoring of coke oven workers by measuring the hydrolysable hemoglobin adducts generated by 1-nitropyrene. Shown are box plots indicating mean and median (waist) values as well as the 95 percentile of controls and workers split into three different job categories.

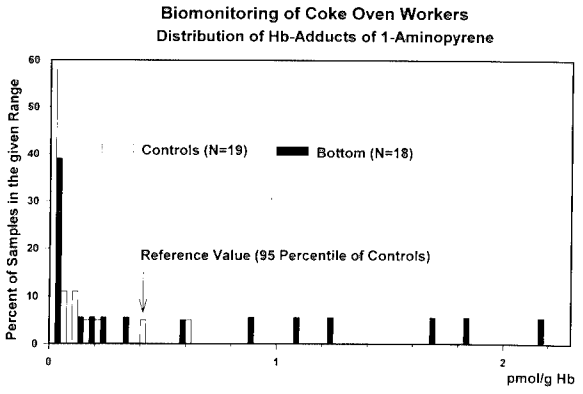


Fig. 3. Distribution of the hydrolysable adduct level generated by 1-nitropyrene. Individuals with adduct levels above the reference value are considered to be site-specifically exposed.

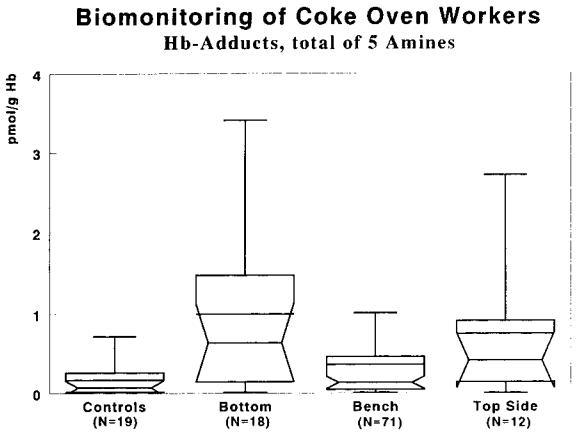


Fig. 4. Biomonitoring of coke oven workers by measuring hydrolysable hemoglobin adducts. Values represent the sum of five specific cleavage products from polycyclic nitro-arenes.

the picture is very similar to that of 5-aminopyrene (Fig. 4).

Biochemical effect monitoring of coke oven workers exemplifies relatively high exposures. Further work is necessary to demonstrate the suitability for routine monitoring of exposures to pyrolysis products by such hemoglobin adducts as compared to the analysis of benzo[*a*]pyrene-DNA or other adducts. An example for low level exposures was studied in individuals living in an area of former production and use of explosives.

4. Monocyclic nitro-arenes as indicators of exposure to explosives

Areas of production and use of trinitrotoluene (TNT)-based explosives from World War I and II are still contaminated considerably with waste material. The appearance of contaminants in groundwater creates a general problem of environmental pollution, but the residents are primarily concerned about the risk involved with direct exposures from their environment [16,17].

2,4,6-TNT was the predominant explosive used, but the waste contains a whole mixture of byproducts from its synthesis. The four most abundant nitro-arenes after 2,4,6-TNT (38% of samples) found in soil from one of those areas are 2,4-dinitrotoluene (2,4-DNT; 16%), 4-amino-2,6-dinitrotoluene (16%), 2-amino-4,6-dinitrotoluene (14%), and 2,6-dinitrotoluene (13%). The content in soil varies from a detection level of 10–50 μg to 1 g/kg dry weight. These chemicals were also found in 73–84% of groundwater samples, except 2,6-DNT.

The five most frequently occurring monocyclic nitro-arenes are mutagenic and either carcinogenic in experimental animals (2,4,6-TNT, 2,4-DNT, 2,6-DNT) or suspected of being carcinogenic. 2,6-DNT is a comparatively strong liver carcinogen in the rat [18]. An increased incidence of liver cancers among munition workers [19] and an increased risk for leukemia in people from a contaminated area [20] have recently been published. The widely discussed questions therefore are: what is the risk of developing cancer for individuals living in such an area? What concentrations of contaminants in soil should be tolerated?

Sophisticated calculations have been performed to estimate the uptake under real life or worst case situations. But the more important question: how much is indeed taken up, has not been approached for a long time. We proposed that this relevant information can be obtained by measuring hemoglobin adducts. In an initial study, we have now analysed blood samples for adduct formation of the five most abundant nitro-arenes mentioned above plus 1,3-dinitrobenzene. With polyfunctional nitro-arenes, the situation is more complicated, since more than one nitroso derivative can be formed metabolically depending on which or how many nitro groups are reduced and further metabolized. In this study, we analyzed only those adducts resulting from metabolites in which only one nitro-group was reduced, i.e. for 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene from 2,4,6-TNT, 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene from 2,4-DNT, 2-amino-6-nitrotoluene from 2,6-DNT, and 1-amino-3-nitrobenzene (3-nitroaniline) from 1,3-DNB.

Blood samples from 34 potentially exposed individuals and 34 controls were processed and analyzed for the six cleavage products mentioned. Except for 1-amino-3-nitrobenzene, all

Table 2
Biomonitoring of monocyclic nitro-arenes from contamination with explosives

Nitro-arene	Cleavage products from Hb-adducts	% Detected	
		Cases ($N = 34$)	Controls ($N = 34$)
2,4,6-TNT	2-Amino-4,6-DNT	50	53
	4-Amino-2,6-DNT	35	27
2,4-DNT	2-Amino-4-NT	35	47
	4-Amino-2-NT	76	76
2,6-DNT	2-Amino-6-NT	91	100
1,3-DNB	1-Amino-3-NB	0	0

the other amines were detected in at least part of the samples (Table 2). Most surprisingly, the amino-nitrotoluenes were found at the highest levels, and there was no difference between potentially exposed and controls (Fig. 5). In particular, 2-amino-6-nitrotoluene was present in all control samples. The amino-nitrotoluenes are therefore not suitable as markers for exposure to explosive wastes. Even the two amino-dinitrotoluenes were detected in controls. There was, however, a difference between exposed and controls, which in the case of 4-amino-2,6-dinitrotoluene is signficant at the $P = 0.05$ level (Fig. 6). This suggests some explosive-related environmental exposure.

As in our first example for coke oven workers, the adduct levels of most of the exposed individuals are within the 95 percentile of controls and the interindividual variation is great. In this instance, only one individual had higher adduct levels than the control with the highest value. The same is true for the sum of the amino-nitrotoluenes. The answer to the first question then is: there might be some explosive-related exposure, but an increment to risk cannot reasonably be derived, because exposures to

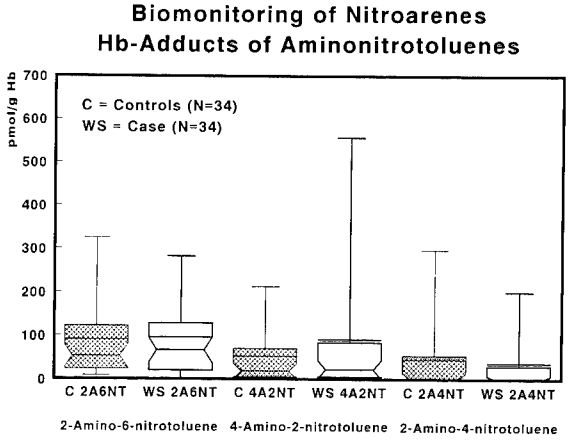


Fig. 5. Biomonitoring of residents living in an area with contaminated soil from explosive wastes and controls. Shown are box plots of values obtained by hydrolysis of hemoglobin adducts from monocyclic nitro-arenes, 2,6- and 2,4-dinitrotoluene. The cleavage products are 2-amino-6-nitrotoluene, 4-amino-2-nitrotoluene, and 2-amino-4-nitrotoluene.

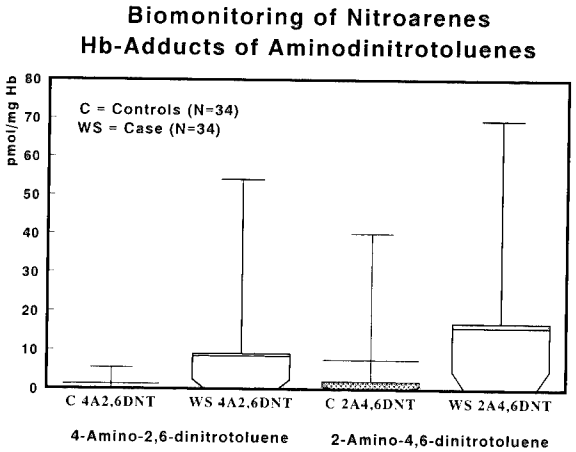


Fig. 6. Biomonitoring of residents living in an area with contaminated soil from explosive wastes and controls. Shown are the results for 2,4,6-trinitrotoluene or its mono-reduction products, 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene, which are also the cleavage products from the hemoglobin adducts. The difference between groups is significant at the $P = 0.05$ level.

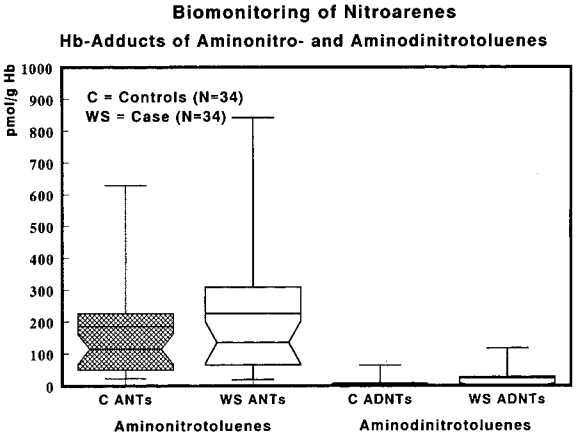


Fig. 7. Comparison of exposures to dinitro- and trinitrotoluenes. The results for the three amino-nitrotoluenes (Fig. 5) and the two amino-dinitrotoluenes (Fig. 6) were added and plotted at the same scale.

DNTs are much higher and not explosive-related (Fig. 7).

One can only speculate about the sources of the high 'background' of nitro-PAH at the present time. Drinking water and air contamination from atmospheric reactions and automobile exhaust come to mind. Before this is clarified, it makes little sense to talk about setting tolerance values for the contaminants in soil and to spend money for costly purification procedures to conform with them. The advice to risk managers on the basis of these results, which should be confirmed in another site, would be to look for the unknown sources of monocyclic nitro-PAH first, then to find out whether they are avoidable, and only thereafter to consider to what extent the soil has to be purified to minimize exposure reasonably.

5. Conclusions

Hemoglobin adducts of nitro-arenes are suitable markers for exposure control, and the method is sensitive enough to detect background levels in real life situations. Unexpectedly, high background levels were found in two different exposure situations which calls for more attention to identify the sources and to examine possibilities for reducing exposure. The general finding of background levels for most studied individual chemicals (11 in the two studies described) emphasizes the need for a more collective approach in risk assessment. It seems necessary to assess the exposure not only of one particular chemical but of classes of related chemicals, like amino- and nitro-arenes. The target dose concept allows a better estimate of the contribution of individual chemicals to the total load and of specific exposures to the general background. It is suggested that exposures to genotoxic carcinogens are minimized according to the ALARA-principle (As Low As Reasonably Achievable), based on the contribution of that specific exposure relative to background and to related chemicals, i.e. the relative risk based on measuring target doses.

Acknowledgements

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Antidotes: benefits and risks

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Abstract

The ability of naloxone and flumazenil to reverse serious toxicity due to their respective agonists is well known. Their adverse effects are minor and few but flumazenil administration may unmask seizures in poisoning with combinations of benzodiazepines and tricyclic antidepressants. The desferrioxamine challenge test is frequently falsely negative and should be abandoned. In addition to being cytoprotective, *N*-acetylcysteine has potent vasodilator and positive inotropic actions which may be partly responsible for its beneficial effect in late-presentation paracetamol overdosage with hepatic damage and failure.

Keywords: Acetylcysteine; Naloxone; Flumazenil; Desferrioxamine; Haemodynamics; Mechanisms

1. Introduction

The antidotes in common clinical use vary according to the toxicological problems of the area of the world under consideration. In the United States, western Europe and Scandinavia the most important are *N*-acetylcysteine (NAC), naloxone and flumazenil which were used in 4.5, 3.9 and 1.4%, respectively of cases reported to the Toxic Exposure Surveillance Scheme of the American Association of Poison Control Centers in 1993 [1]. No other antidote has application in more than 1% of poisonings but of these, desferrioxamine deserves mention because of its role in preventing childhood deaths from acute iron poisoning. Agents designated antidotes are used in the treatment of confirmed poisoning, in the diagnosis of poisoning and, frequently, on a 'nothing to lose' basis. They are widely held to be inherently safe but unfortunately, this is not always the case. Antidotes commonly have dose-related adverse effects, may cause hypersensitivity reactions and may lead to misdiagnosis. Fetotoxicity and teratogenicity are concerns when they are to be given to pregnant women. In every case the potential benefits of the use of an antidote have to be weighed against the risks.

2. Naloxone

The response of pure opioid overdosage to administration of naloxone remains one of the most dramatic events in clinical medicine. Within a minute or so coma is completely reversed, the respiratory rate increases and the respiratory minute volume frequently but transiently exceeds normal. It is less well appreciated that the blood pressure also rises transiently. Naloxone has very few adverse effects. These include an acute withdrawal syndrome in individuals dependent on opioids, and pulmonary oedema and

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ventricular fibrillation in non-dependent and otherwise healthy individuals. Fortunately the incidence of such reactions is extremely low and not sufficient to contraindicate the use of the antidote in appropriate, and even in inappropriate, situations. Naloxone has also been claimed to reverse (at least to some extent) the toxicity of ethanol, benzodiazepines, clonidine and captopril but the evidence is inconclusive [2]. There is also some anecdotal evidence to indicate that it may have a beneficial effect on the haemodynamic consequences of poisoning with dextropropoxyphene and its major metabolite, norpropoxyphene, increasing the cardiac output and left ventricular stroke work index and partially reversing the dilatation of the systemic vascular bed caused by these drugs [3]. The mechanism is unclear but angiotensin II release subsequent upon removal of the inhibitory effects of endorphins by naloxone has been postulated.

3. Flumazenil

Overdosage with benzodiazepines alone seldom leads to such serious consequences that flumazenil is an indispensable component of treatment. It is commonly used diagnostically in patients who are unconscious due to unknown drugs and in others in whom the cause of coma is uncertain. In such situations it may produce such observable clinical improvement that there is no doubt that benzodiazepines are contributing to the patient's clinical state. It may therefore obviate the need for CT scanning of the brain in some cases and has the therapeutic advantages of significantly reducing the number of patients requiring endotracheal intubation, assisted ventilation and bladder catheterisation [4]. The incidence of adverse reactions to flumazenil is significant. They occurred in nine out of 53 unconscious patients, only 36 of whom had benzodiazepines present in their serum [4]. However they were minor in all but one patient who became hypotensive, the systolic blood pressure falling from 100 to 60 mmHg. Seizures and arrhythmias were not observed.

Concern has been expressed in respect of the safety of flumazenil administration to individuals who have ingested combined overdoses of tricyclic antidepressants and benzodiazepines. The disadvantage of the combination is that the latter will potentiate the CNS depressant actions of the former but they may also have a more important advantage by reducing the frequency of seizures induced by the antidepressants. Administration of flumazenil may then remove the protective effect of the benzodiazepines and unmask seizures. The evidence that this occurs in practice is again anecdotal but the consequences for individual patients are potentially disastrous. Seizures are associated with hypoxaemia and metabolic acidosis and there is clinical suspicion that they may trigger lethal arrhythmias. The ability of flumazenil to precipitate seizures in animals poisoned with tricyclic antidepressants has been clearly demonstrated [5]. It would therefore seem that flumazenil should not be given to patients who have ingested benzodiazepines in combination with tricyclic antidepressants.

4. Desferrioxamine (DFO)

Acute iron overdosage is mainly a problem of young children, approximately 75% of incidents occurring in those under the age of 6 years [1]. Serious and fatal [1,6–8] poisonings are usually associated with ingestion of adult iron preparations and animal studies have clearly shown that DFO reduces mortality. Unfortunately, it has potentially serious adverse reactions in therapeutic doses including anaphylaxis and hypotension when intravenous infusion rates exceed 15 mg/kg body weight per hour. Indiscriminate use is therefore unjustified. Selection of appropriate patients for treatment with DFO is difficult, there being no single parameter of value. Generally a decision to give the antidote is based on a combination of the clinical condition of the patient (especially the presence of shock or impairment of consciousness) and the serum iron concentration. The latter is not always available on an emergency basis and some years ago the desferrioxamine challenge test was introduced to

identify patients with circulating free iron (i.e. serum iron concentrations in excess of the total iron binding capacity). The challenge involves giving a single intramuscular dose of DFO (50 mg/kg body wt. up to a maximum of 1 g) and observing the colour of the urine. In patients in whom DFO has chelated free iron, the chelation complex, ferrioxamine, is excreted in the urine turning it an orange/red colour – so called vin rosé. While the principle underlying the test is sound, there have been problems in practice. A number of studies [9–11] have shown that approximately 70% of patients with serum iron concentrations exceeding expected TIBC who were challenged with DFO failed to develop the diagnostic change in urine colour. The reasons are unclear. It may simply be that the concentration of ferrioxamine in the post-challenge urine is too low to produce a colour change detectable to the naked eye. The pH of the urine does not seem to be a relevant factor. The desferrioxamine challenge test should therefore be abandoned.

There have been no controlled trials of DFO in acute iron poisoning in humans. Indeed the toxicokinetics of iron after poisoning and the mechanisms of action of DFO have been inadequately studied. Theoretically, 100 mg of DFO could bind approximately 8.5 mg of ferric iron which should then be excreted in the urine as ferrioxamine. However, the beneficial effects of DFO cannot always be explained so simply as illustrated by the findings in a young adult female who presented with haematemesis and grey watery diarrhoea and had a serum iron concentration of 13.4 mg/l (240 μmol/l) 6 h after ingestion of 2.4 g of elemental iron. She was given DFO in conventional doses but over the next 3 days only 50.7 mg (0.91 mmol) of iron, the equivalent of the iron content of only one tablet of ferrous sulphate, was recovered from the urine [12].

5. *N*-Acetylcysteine (NAC)

The alarming increase in the numbers of individuals consuming overdoses of paracetamol (acetaminophen) over the past 30 years has arguably made NAC the single most important antidote in developed countries. It is certainly the one which is most widely used [1] and several controlled studies in poisoned patients have demonstrated its ability to prevent the liver damage caused by the intermediary metabolite of paracetamol metabolism, *N*-acetylbenzoquinoneimine [13]. The incidence of acute renal tubular necrosis can also be reduced. These benefits are achieved whether NAC is given orally or intravenously provided it is given sufficiently early in the course of poisoning [13]. Later than 10 h after ingestion of paracetamol, the ability of NAC to prevent hepatic necrosis declines.

Adverse effects occur in up to 10% of patients receiving the intravenous NAC regime favoured in the UK, Australia and parts of Europe. These reactions have been termed 'anaphylactoid' because they include flushing, rash, itching, angioedema and bronchospasm [14]. However, patients given overdoses of NAC of between 1.5 and 10 times normal doses experience similar features although hypotension and flushing are probably more common while rashes and pruritus are less so. Almost invariably adverse reactions to intravenous NAC occur within 2 h of the start of the infusion [15], the time at which plasma concentrations of the antidote are maximal [16]. There is therefore doubt as to whether they are due to hypersensitivity or are dose-related. Skin testing of a small number of patients who experienced reactions to NAC show that there is a threshold for weal formation after intradermal injection of the commercial formulation and that responses only occur with high concentrations. The study concluded that the mechanism of adverse reactions was 'pseudo-allergic' rather than immunological [15].

When NAC was introduced it was feared that its administration to patients presenting more than 15 h after paracetamol overdosage might increase the risk of hepatic encephalopathy and it was therefore held to be contraindicated at this late stage. However, desperate clinicians faced with ever increasing numbers of predominantly young people with paracetamol-induced fulmin-

ant hepatic failure (FHF) have gradually extended the period over which NAC is given and the concern that the antidote might exacerbate encephalopathy has failed to materialise. Indeed, in one study of paracetamol-induced FHF patients given NAC until recovery from encephalopathy or death, mortality was significantly reduced and the incidence of cerebral oedema and hypotension requiring inotropic support was lowered [17]. Moreover, the benefits were achieved safely and without adverse reactions. The mechanism by which late NAC administration protects the liver has been the subject of much speculation.

Haemodynamic investigation of patients in FHF induced by paracetamol has shown that NAC increases oxygen delivery, oxygen consumption and the oxygen extraction ratio. This is partly due to an increase in the cardiac index (CI) and mean arterial blood pressure (MABP) despite a decrease in systemic vascular resistance (SVR) [18]. Such effects may therefore be sufficient to account for the benefits of NAC late in the course of paracetamol poisoning.

While it is desirable to know the haemodynamic effects of NAC on hepatic blood flow in paracetamol-induced FHF, placement of catheters in the femoral and hepatic veins and intravenous injection of indocyanine green is required. Clearly such studies pose ethical difficulties in critically ill patients but have been carried out in patients in a stable phase of cirrhosis [19]. The previously observed effects of NAC on CI and oxygen delivery to tissues have been confirmed. Despite this, oxygen consumption and the oxygen extraction ratio were not increased probably due to either shunting of blood through the lungs or ventilation/perfusion mismatching. Vascular resistance fell both in the systemic and pulmonary circulations but MABP was maintained indicating that the force of myocardial contraction must have increased. Calculation of left ventricular stroke work index confirmed that this was indeed the case. NAC therefore has an important positive inotropic action [19] which may explain the reduced need for inotropes and renal support in the NAC-treated FHF patients [17]. Estimated liver blood flow and portal venous pressure did not change in the cirrhotic patients but this would not exclude the possibility that NAC might induce beneficial changes in hepatic blood flow in those with FHF.

Studies of forearm blood flow in cirrhotic patients and healthy volunteers [20] demonstrate that NAC is a local vasodilator in both groups and is as potent as bradykinin. The mechanism by which it achieves this effect is unclear. It may be secondary to stimulation of the endothelial receptor, direct stimulation of nitric oxide synthetase or a direct effect on vascular smooth muscle cells. Further studies using L-*N* monomethyl arginine (L-*N*MMA, a specific nitric oxide synthetase inhibitor) and bradykinin antagonists may clarify this mechanism further.

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Modifying toxicokinetics with antidotes

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Abstract

Five approaches may be described through which antidotes can modify toxicokinetics: (1) Decreased bioavailability of the toxins; (2) Cellular redistribution of the toxin in the organism; (3) Promotion of elimination in an unchanged form; (4) Slowing of metabolic activation pathways; (5) Acceleration of metabolic deactivation pathways. However, the ability to modify toxicokinetics with a new treatment, while demonstrating an understanding of the mechanism of action, must never be construed to be, in and of itself, the goal of therapy. The ultimate evaluation of an antidote modifying toxicokinetics is strictly clinical.

Keywords: Antidote; Toxicokinetics; Toxicodynamics; 4-Methylpyrazole; *N*-acetylcysteine; *Digitalis*

1. Introduction

Toxic substances act principally by three mechanisms: physical (e.g. foam from shampoo), chemical (e.g. acid burns), or biological (e.g. *Digitalis*). In some cases (e.g. hydrofluoric acid) the mechanisms of toxicity are multiple. However, the vast majority of poisonings result from a biological mechanism. For toxicants which act by this third mechanism, the poison must be absorbed, distributed and must reach a critical concentration at the cellular target (toxicokinetics). Such a critical concentration results in cellular modifications, reversible or irreversible, which may ultimately be translated into signs and symptoms of clinical illness (toxicodynamics). The classic treatment paradigm in clinical toxicology includes: (1) Supportive treatment; (2) Prevention of absorption of toxic compounds; (3) Enhancement of their elimination; (4) Specific treatments, including antidotes. However, this description of general principles of treatment of poisoning does not take into account the importance of this duality of toxicokinetics and toxicodynamics, encountered in the majority of poisonings.

In all dictionaries throughout the world, antidotes are defined as 'a remedy to counteract the effects of a poison'. However, such a global definition does not help us, as physicians, to know what should be beneficial for our patients. Thus, we recently proposed a more conservative definition of antidotes [1] which we have now expanded: 'An antidote is a drug whose mechanisms of action have been determined, which is able to modify either the toxicokinetics or the toxicodynamics of the poison and whose administration to the poisoned patient reliably induces a significant benefit.' As such it appears that many drugs with unproven efficacy (Fuller's earth in

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paraquat poisoning) in human poisoning or unknown mechanism (diazepam in chloroquine poisoning) should not presently be referred to as 'antidotes'. Such a restrictive definition should help to distinguish between drugs which are merely adjunctive therapy from those which are true antidotes.

Many classifications of antidotes have been proposed, none completely satisfactory [2]. Antidotes may be classified as a function of their mechanism of action into eight categories which cover the toxicodynamic and toxicokinetic aspects of intoxications (Table 1). Toxicokinetic treatment includes those antidotes that decrease the concentration of the toxic compound at the level of the cellular target. Toxicodynamic treatment involves antidotes which modify clinical symptomatology without affecting the concentration of the toxic compound at the level of the cellular target. In general, toxicodynamic treatment may dramatically improve immediately life-threatening symptoms but is devoid of effect on the duration of the poisoning. It should be noted that most supportive treatments work on a toxicodynamic basis. Toxicokinetic treatment such as gut decontamination, on the other hand, tends to either prevent or decrease the duration of the intoxication, but may not have any immediate effect on clinical symptoms. There are notable exceptions to these generalities. *Digitalis* antibodies which are a toxicokinetic therapy provide almost immediate clinical improvement while decreasing free *Digitalis* tissue concentration. In contrast, both ethanol and 4-methylpyrazole actually prolong the duration of exposure to the native toxin (ethylene glycol and methanol) but decrease the exposure to toxic metabolites (glycolate and formate). On the other hand, among acute poisonings causing life-threatening cardiovascular collapse, which has been shown to impair the elimination of toxins [3], it stands to reason that toxicodynamic treatments which improve hemodynamic status and tissue perfusion in the liver and kidney may be expected to decrease the duration of intoxication.

Decreasing the amount of toxin in the body should, in theory, improve the patient's condition. However, there are a number of factors which impede such a simple relationship (Fig. 1). The actual immediate clinical benefit of reduction of body burden of a toxin is dependent on (1) the amount of toxin present in the body in excess of that required to produce a toxic effect, (2) the slope of toxicity, (3) the ratio of the amount of toxin removed to the dose required to

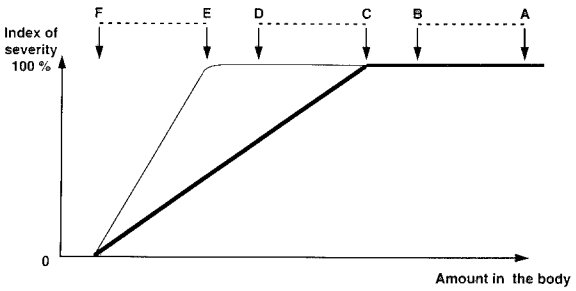


Fig. 1. Relationship between the clinical severity of the poisoning and the amount of toxin in the body in two types of intoxication, one with a gentle slope of toxicity (1) and the other with a steep one (2). The clinical effect of the removal of a fixed amount of toxin depends on the ratio of the amount of toxin removed to the dose required to produce a toxic effect.

Table 1
Classification of antidotes and chelators

Treatment	Effect
Toxicokinetic treatment	1 Decreased bioavailability of the toxins
	2 Cellular redistribution of the toxin in the organism
	3 Promotion of elimination in an unchanged form
	4 Slowing of metabolic activation pathways
	5 Acceleration of metabolic deactivation pathways
Toxicodynamic treatment	6 Competitive or non-competitive displacement of the toxin from its binding site
	7 Bypass of the binding of the toxin to the receptor
	8 Correction of peripheral effects of the toxins (including supportive treatments)

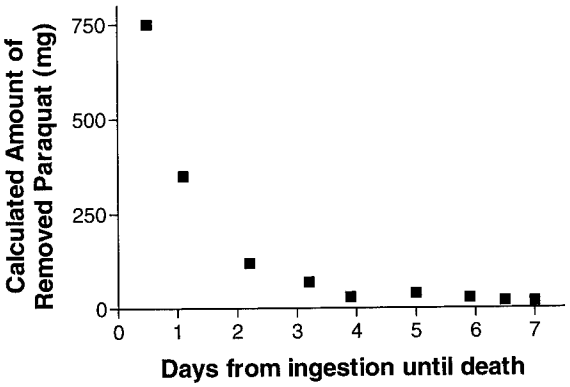


Fig. 2. Efficacy of hemoperfusion/hemodialysis and time to death in nine paraquat poisoning patients. The greater the amount of paraquat removed, the faster the patient succumbed. From Bismuth [20] with permission.

produce a toxic effect, (4) the delay in onset of treatment. These factors appear critical for toxins inducing irreversible lesions. Fig. 2 shows that in cases of acute paraquat poisoning treated by extracorporeal methods the greater the quantity of paraquat removed the more rapidly the patient died. We interpret this seeming paradox as follows: the total amount of toxin removed has no clinical significance per se. In this case, increased removal of toxin is consistent with a body burden far in excess of the amount required to produce death. Thus, we conclude that modifying toxicokinetics with a new treatment, while demonstrating an understanding of the mechanism of action, must never be construed to be, in and of itself, the goal of therapy.

Thus, it is necessary that we examine in further detail what constitutes a 'significant benefit' in antidotal therapy. It is necessary but not sufficient that an antidote improves the toxicokinetics and/or toxicodynamics in poisoning. To consider an antidote effective in human beings requires that the course of illness and the outcome are favorably modified. Significant benefits may be realized not only in improved prognosis but also as decreased duration or cost of hospitalization or decreased requirements for invasive procedures. Very few antidotes have been investigated in this light (Table 2). In fact, the evaluation of antidotes in humans has been constrained by numerous factors. First of all, for a number of potentially lethal toxins, there are an insignificant number of patients encountered in any one treatment center to permit an assessment of efficacy on a prospective basis. In addition, increasing administrative and legal hurdles limit testing of new antidotes and even old ones. It must be taken into account that many acute intoxications induce rapid onset of severe symptoms requiring emergency treatment. In this particular clinical setting, the early administration of toxicokinetic antidotes is of utmost importance if they are to be effective. Requirements for informed consent, frequently impossible in the poisoned patient, may need to be waived.

Five approaches may be described through which antidotes can modify toxicokinetics: (1) Decreasing the bioavailability of the poison; (2) Enhanced elimination of unchanged poison

Table 2
Attempt at classification of toxicokinetic antidotes according to the derived benefit in human poisoning

Derived benefit	Proven efficacy	Possible efficacy
Life-saving	*N*-Acetylcysteine Fab fragments Oxygen Desferoxamine	Chelating agents Cyanide antidotes Methylene blue
Decreased duration of hospitalization	?	Oxygen Activated charcoal (?) *Digitalis* Fab fragments (?)
Decreased cost of hospitalization	?	?
Decreased requirements for invasive procedures	Activated charcoal 4-Methylpyrazole DMSA	Whole bowel irrigation

through natural means; (3) Slowing of metabolic activation; (4) Accelerating an inactivating metabolism; (5) Promoting an extracellular redistribution of toxicant.

2. Decreasing the bioavailability of the poison

Gastric lavage should no longer be considered as a routine management procedure in poisoned patients, as it has a very limited application. Currently, gastrointestinal decontamination is accomplished by a pharmacological intervention (Table 3).

A single dose of activated charcoal appears capable of preventing the absorption of many toxic compounds, providing that both the delay between drug ingestion and charcoal administration is short and the ratio of the dose of activated charcoal to the ingested dose of the toxicant is nearly 10:1. Furthermore, repeated doses of activated charcoal may increase the endogenous clearance of many toxic compounds through 'gastrointestinal dialysis' [4]. However, the actual clinical benefit of single and repeated doses of activated charcoal for many poisonings remains to be determined. Due to its safety, repeated doses of activated charcoal should be recommended in various intoxications [5] such as phenobarbital, theophylline (in which case, repeated doses of activated charcoal should be considered as viable alternative to hemoperfusion), phenytoin, carbamazepine, and salicylates (Table 3). One randomized clinical study compared the effect of repeated doses of activated charcoal to a single dose in phenobarbital poisoning. The half-life of phenobarbital in plasma was significantly shortened while the duration of intubation did not differ [6].

3. Enhanced elimination of unchanged poison through natural means

3.1. Chelating agents for heavy metals (Table 4)

One of the earliest chelating agents was dimercaprol (BAL). Recently, two other related drugs have been introduced, dimercaptosuccinic acid (DMSA) and dimercaptopropane sulfonic acid (DMPS) that possess the same dithiol

Table 3
Decreasing the bioavailability of the poison

Antidotes	Toxicants
Activated charcoal	'All' but toxic alcohols, caustics, iron, lithium[a]
Whole bowel irrigation	Body-packers, iron, theophylline[b], verapamil[b], lead, zinc sulfate
Prussian blue	Thallium, cesium
Calcium salts	Fluoride
Magnesium sulfate	Barium (soluble forms)

[a] Also increase the elimination of digitoxin, phenobarbital, carbamazepine, phenylbutazone, dapsone, methotrexate, nadolol, theophylline, salicylate, cyclosporine E, propoxyphene, amitriptyline, and nortriptyline.
[b] Sustained-release preparations.

Table 4
Chelating agents used in human poisonings

Chelating agents	Toxicants
Disodium calcium ethylenediamine tetracetate (EDTA Na_2Ca)	Lead, zinc, cadmium (highly controversial)
Diethylenetriamine pentacetate (DTPA)	Plutonium, americium
Dimercaptopropanol (BAL)	Arsenic (inorganic and organic [lewisite]), mercury salts, lead[a]
Dimercaptopropanesulfonic acid (DMPS)	Arsenic, lead, mercury (organic and inorganic)
Dimercaptosuccinic acid (DMSA)	Lead, organic and inorganic mercury, arsenic
Penicillamine	Copper overload, lead, arsenic, mercury
Desferoxamine	Iron, aluminium

[a] In addition to EDTA $CaNa_2$.

chelating group, while the whole molecules are more hydrophilic [2,7]. Unlike dimercaprol, these two recent drugs can be used orally and have a better therapeutic index than BAL. A considerable amount of work has been carried out on reaction of the chelating agents with metals, and it is possible to predict the efficacy of particular chelating agents in particular metal poisonings on the basis of the affinity constant of the metal and chelator. It should be noted that the precise mechanisms of action by which the chelating agents work are not fully understood. It seems likely that the beneficial action of the chelating agents is probably a combination of effects, detoxification by complexation, mobilization, and elimination. Thus, the affinity constant cannot be the sole property that determines the efficiency of a particular chelating agent for a particular metal. Indeed, the properties of an ideal chelating agent have been defined by Yokel and Kostenbauder [8] and should include a high affinity for the metal of interest, be sufficiently water-soluble to take by mouth, and be sufficiently lipid-soluble to distribute to sites of accumulation of the metal. Thus, Marrs and Bateman [2] concluded that if this were generally the case, partition studies would clearly improve the predictive value of in vitro studies of the chelating agents.

3.2. Oxygen works in carbon monoxide poisoning by two mechanisms

A high partial pressure of oxygen displaces carbon monoxide from the iron of hemoglobin, myoglobin and various enzymes in a competitive manner. Simultaneously, a high partial pressure of oxygen increases the pulmonary elimination of carbon monoxide. It should be outlined that it is unusual for antagonistic antidotes to modify the kinetics of the toxicant. The relative importance of these two mechanisms in the treatment of human poisoning is not known. In our day-to-day experience, many carbon monoxide poisonings found unconscious awaken within a few minutes while breathing pure oxygen. This rapid improvement seems incompatible with what is known about the elimination half-life of carboxyhemoglobin. It must be emphasized that in comparison with what is known of the kinetics of other drugs, the knowledge of the kinetics of carbon monoxide is very limited.

4. Slowing of metabolic activation

The toxicity of both methanol and ethylene glycol (EG) results from their metabolism to more toxic metabolites by liver alcohol dehydrogenase: formate (methanol), glycolate and oxalate (ethylene glycol) [9]. The conventional treatment of these life-threatening poisonings includes gastric decontamination, massive amounts of sodium bicarbonate, blockade of the metabolism of toxic alcohols by ethanol, and hemodialysis. However, the indications for the respective parts of this treatment regimen remain a matter of debate, as ethanol treatment may be inefficient when doses used are too low or result in side-effects when administered in excess. 4-Methylpyrazole (4-MP) is a very potent inhibitor of alcohol dehydrogenase (ADH) activity in various species, including humans. 4-MP has a profound inhibitory effect on the oxidation of both methanol and EG in monkeys and dogs, respectively [10].

Because 4-MP is an inhibitor of ADH rather than a competitive substrate like ethanol, it has been suggested as having greater value than ethanol in treating EG poisoning. 4-MP has other therapeutic advantages over ethanol: it does not exert CNS depressant activity and it has a longer duration of action than ethanol because of slower elimination. Studies in animals and healthy volunteers have shown that repeated doses of 4-MP are safe, in contrast with its parent compound pyrazole. Recently, 4-MP treatment has been tried in a limited number of acute human ethylene glycol poisonings admitted early after ingestion. 4-MP was found to be both efficient and safe. Both intravenous and oral regimens of 4-MP, dosed every 12 h until plasma EG became undetectable, were able to block the metabolism of EG, avoiding the requirement for hemodialysis in patients with high plasma EG levels and normal renal function [11]. Normal renal function allows the elimination of unchanged EG. 4-MP appears to be a very promis-

ing antidote allowing a less invasive treatment of early-admitted EG poisoning.

To our knowledge, the efficiency of 4-MP in acute human methanol poisoning has not been assessed. There is a large body of knowledge supporting the use of 4-MP in methanol poisoning. However, some differences between EG and methanol poisonings preclude drawing any inference from the efficiency of 4-MP in EG poisoning. Indeed, in patients with normal renal function, the renal clearance of EG is in the range of 20–40 ml/min with an elimination half-life of about 12 h. In contrast, unchanged methanol is eliminated very slowly, mainly by the renal route, its renal clearance being about 1–2 ml/min, corresponding to a plasma half-life of about 40–50 h. Thus, theoretically, blocking methanol metabolism with an inhibitor requires consideration of hemodialysis as the safety of repeated doses of 4-MP has only been demonstrated to 96 h, and more prolonged treatment has not been assessed.

4-MP has also been shown to be useful in treating disulfiram-like reactions. These manifestations result from the ingestion of ethanol in a patient having a blockade of aldehyde dehydrogenase by disulfiram or related compounds, thus allowing the accumulation of acetaldehyde, the toxic metabolite. Disulfiram reactions can induce life-threatening poisoning due to the accumulation of acetaldehyde in blood. The conventional treatment of this occasional poisoning includes supportive treatment and at times β-blocking agents in case of vasoplegic shock. 4-MP was shown to block acetaldehyde accumulation and reverse the toxic manifestations in an animal model of disulfiram reactions. Similarly, a single intravenous 7 mg/kg dose of 4-MP reversed the signs and symptoms in a patient suffering from disulfiram reaction [12].

5. Accelerating an inactivating metabolism

N-Acetylcysteine (NAC) is a life-saving antidote in paracetamol poisoning. Clinical studies suggest that, depending on the delay between ingestion and treatment, several mechanisms of action may come into play. It is fascinating to note that the antidote in the early phase does not impede the production of the toxic metabolite *N*-acetyl-*para*-benzoquinonimine (NRPQI), but rather scavenges the toxic radical as it is produced. However, the mechanisms of action of *N*-acetylcysteine are quite complex, involving several metabolic pathways. Indeed, NAC enhances synthesis of glutathione and production of sulfate. Furthermore, it encourages the reduction of NAPQI to paracetamol [13]. This latter mechanism of action would be associated with an increase in the elimination half-life of paracetamol, which has been reported in severe poisoning but only in the setting of severe hepatic damage.

Thiosulfate is the cosubstrate of rhodanese, the hepatic enzyme, which transforms the cyanide ion to thiocyanate by incorporation of an atom of sulfur. Thiosulfate is a remarkably effective antidote in intoxications by compounds which slowly liberate cyanide such as sodium nitroprussate. In our experience it seems also beneficial during the acute phase of aliphatic nitrile poisonings in combination with hydroxocobalamin. We believe that hydroxocobalamin is the drug of choice to treat the acute manifestations of aliphatic nitrile poisonings and should be administered rapidly, while sodium thiosulfate has a more prolonged benefit and should be administered as a continuous infusion in the prevention of recurrent cyanide poisoning. As in the case of NAC, sodium thiosulfate does not inhibit hepatic production of the toxic metabolite (in this case cyanide) but provides for its rapid elimination.

6. Promoting an extracellular redistribution of toxicant

Among the antidotes acting by induction of an extracellular redistribution of the toxicant are the immunotoxicotherapeutic agents and several antidotes to cyanide, namely hydroxocobalamin and the methemoglobin-forming agents.

The last two decades have seen a rise in importance of immunotoxicotherapy. Two recent improvements in immunotherapy are representative: *Digitalis* and colchicine poisonings. The

development of *Digitalis* Fab fragments represented a major improvement in immunotherapy, greatly improving its safety in comparison with antivenoms. Fab fragments work by inducing (1) extracellular redistribution, (2) sequestration of the toxin in the extracellular space, and (3) renal elimination of a small molecular weight antibody-toxin complex. This therapeutic approach is limited by the ratio of the mass of the antidote to that of the poison. The practice of immunotoxicotherapy with *Digitalis* Fab has been to reverse the poisoning in an equimolar fashion. With drugs such as *Digitalis* which are toxic in minute amounts such an approach is feasible, but expensive. Our experience in *Digitalis* poisoning suggests that partial neutralization can effectively convert a fatal intoxication to a non life-threatening one. These preliminary results must be confirmed. The management of *Digitalis* intoxication continues to rely on supportive care of the patient, and early gastrointestinal decontamination. Due to their cost, *Digitalis*-specific Fab antibody fragments are recommended only in severe poisonings unresponsive to conventional treatment. However, in spite of the current availability of digoxin-specific Fab fragments, severely poisoned patients still die. Recently, in one large series of cardiac glycoside poisoning the analysis of the causes of death revealed that the main obstacles to the success of Fab fragments were pacing-induced arrhythmias and delayed or insufficient administration of Fab [14]. Indeed, the iatrogenic accidents of cardiac pacing were frequent (14/39) and often fatal (5/39). In contrast, immunotherapy was not associated with any serious side-effects (0/28) and was safer than pacing. Thus, cardiac pacing appears of limited value and even harmful in the treatment of acute *Digitalis* poisoning. These results suggest that Fab fragments should be first-line therapy during acute *Digitalis* intoxication. Fab treatment should be recommended in patients exhibiting either severe ventricular arrhythmias or poor prognostic factors. Poor prognostic factors of acute *Digitalis* poisoning include: (a) advanced age, (b) heart disease, (c) male sex, (d) high degree atrioventricular block or bradycardia refractory to atropine, and (e) hyperkalemia. In our experience, early treatment with *Digitalis* Fab fragments, while expensive, tends to decrease the duration of hospitalization in a critical care unit, offsetting the cost of the antidote (14).

Digitalis Fab fragments demonstrated that immunotoxicotherapy may be very effective against membrane-level toxins. The recent development of colchicine-specific Fab fragments has revealed the exciting proposition that immunologic fragments which remain in the extracellular space can reverse the effect of intracellular toxin [15]. The reversal of colchicine toxicity, at least in the case of delayed treatment, has not been global, however. While there is dramatic improvement in cardiovascular function, the effect on the hematopoietic system persists.

As was previously mentioned with regard to digoxin, partial neutralization presents numerous advantages in immunotoxicotherapy, due to the limited availability and high cost of the antibody products. This concept of partial neutralization has been further exploited in the form of colchicine-specific Fab fragments. As animal experiments demonstrated efficacy of partial neutralization of colchicine [16], the first human intoxication was treated with a substoichiometric dose of colchicine-specific Fab fragments [15].

Cyanide poisoning, more frequent than previously suspected [17], remains life-threatening. Supportive treatment in association with oxygen has been reported effective in acute cyanide poisoning. It should be noted that all presently available cyanide antidotes except for oxygen work on a toxicokinetic basis, specifically by the reduction of free cyanide at the tissue level. The efficacy of these other antidotes has not been unequivocally proven in man. Hydroxocobalamin and methemoglobin-forming agents act in proximity to the sites of injury, principally the brain and heart. Thiosulfate on the other hand provides a substrate for hepatic metabolism thus acting at a distance from the target organ. The transformation of hydroxocobalamin to cyanocobalamin has been demonstrated in vivo in acute cyanide poisoning [18]. This transformation is rapid and strictly related to the concentration of blood cyanide up to 1 mg/l. It appears to be

well tolerated in man even in the complex setting of smoke inhalation [19]. The administration of hydroxocobalamin is associated with a rapid and constant improvement in systolic blood pressure alleviating the need for catecholamine support. Whether it provides additional benefits remains to be seen.

7. Non-specific modulation of the toxicokinetics

All drugs that improve the hemodynamic status may also improve the perfusion of the gastrointestinal tract, liver and kidney. Thus, depending on the amount of toxin remaining in the gut, the net result may be either worsening of the clinical status due to increased absorption or improvement by promoting elimination of the toxicants.

8. Conclusion

Many possibilities exist to modify the toxicokinetics of a toxicant by specific antidotes. However, an efficient antidote is merely the translation of what we know of the pathophysiology of the poisoning. The introduction of a new antidote is the prize awarded for the molecular approach to the treatment of acute poisoning. The antidotal approach to the toxicokinetic treatment of human poisoning is less invasive and more easily administered than conventional supportive therapy, but requires more sophisticated reasoning. The ability to modify toxicokinetics with a new treatment, while demonstrating an understanding of the mechanism of action, must never be construed to be, in and of itself, the goal of therapy. The ultimate evaluation of an antidote modifying toxicokinetics is strictly clinical. Further clinical assessment of toxicokinetic treatments on a more stringent scientific basis using prospective methods must be promoted.

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Toxicology Letters

Modification of cyanide toxicodynamics: mechanistic based antidote development

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Abstract

Cyanide's actions are complex and cannot be attributed solely to inhibition of oxygen utilization. Recent mechanistic studies show that cyanide inhibits multiple enzymes and alters several vital intracellular processes to produce the intoxication syndrome. By understanding the intracellular targets and the mechanisms underlying the toxicity, it is proposed that more effective antidotal regimens can be achieved. A mechanistic based, multi-step in vitro model was developed for screening potential cyanide antidotes. A series of compounds was screened for their ability to reverse the effect of cyanide on six neurochemical markers in the PC12 cell line (neuronal cell model). Each compound was assigned a composite score based on the six assays; several compounds were identified which then exhibited efficacy in animal testing. Additional mechanistic based studies show that antioxidants and nitric oxide generators have promise as anti-cyanide agents. It is concluded that mechanistic based antidote design can be used to identify new compounds for testing in animal models.

Keywords: Cyanide antidotes; PC12 cell; Catalase; Nitric oxide; Antioxidants; Neurotoxicity

1. Introduction

Cyanide produces a rapid onset of toxicity which must have vigorous and immediate treatment to prevent morbidity and mortality. Immediate symptoms include convulsions, seizures, respiratory failure and cardiovascular collapse, which were thought to result from inhibition of cytochrome oxidase [1]. Recent studies indicate cyanide's actions are complex and cannot be attributed solely to inhibition of oxygen utilization [2]. By understanding in detail the mechanism of toxicity, mechanistic based design of more effective antidotal regimens can achieved.

Development of effective antidotes for cyanide has been an area of active work for over 100 years [1]. The first compounds to be used were developed by empirical observations in which animal studies were used to find compounds that would reverse toxicity. In 1888, Pedigo showed that amyl nitrite was an effective antagonist, followed by the observation by Lang in 1894 that sodium thiosulfate was effective. Also the use of cobalt compounds as cyanide complexing agents was introduced in 1894. In 1933, K.K. Chen used the combination of nitrite and thiosulfate as a highly efficacious antidotal regimen. The mechanism underlying the activity of these antidotes was thought to be understood. However, today, it is becoming apparent that the mechanism of

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both the antidotes and cyanide is more complex than originally proposed [2,3].

2. Mechanisms of cyanide intoxication

Cyanide intoxication is the result of a complex series of effects, with the primary sites of action in the CNS and cardiovascular system. A number of enzymes and cellular processes are altered by cyanide, and the manifestations of toxicity are the result of these multiple actions. When considering mechanisms, cyanide toxicity can be viewed from two perspectives: acute life threatening and subacute or post intoxication sequela (Table 1). In acute intoxication, cyanide produces a rapid inhibition of cytochrome oxidase, resulting in an energy deficit within the target tissue [4]. Additionally, a number of other enzymatic processes are inhibited and may contribute to toxicity [5,6]. This includes the antioxidant defense enzymes (catalase, superoxide dismutase and glutathione peroxidase). Cyanide is also a potent stimulator of neurotransmitter release, both in the CNS and in the peripheral nervous system [7]. All of these events contribute to the acute toxic syndrome and represent potential sites of antidotal action. In toxicity resulting from subacute exposure or in the post intoxication sequela characterized by CNS pathology, a number of events contribute to the toxicity [8]. The prolonged energy deficit can lead to a loss of cellular calcium ion homeostasis resulting in activation of calcium signalling cascades and eventually cell injury. Within the CNS the result is damage to select brain areas.

Table 1
Mechanisms of cyanide intoxication

Acute life threatening
– Inhibition of cytochrome oxidase
– Inhibition of multiple enzymes/processes
– Global stimulation of CNS
Subacute or post intoxication sequela
– Prolonged energy deficit
– Loss of ionic homeostasis
– Activation of signalling cascades
– Oxidative stress
– Necrosis and/or apoptosis

Table 2
Classes of cyanide antidotes

Class	Prototype antidote
Detoxification	Sulfane Sulfur Cpds (thiosulfate)
	Rhodanese
Scavengers	MetHb formers (nitrite, DAMP)
	Alpha-ketoglutarate
	Sodium pyruvate
	Cobalt Cpds
	Methemoglobin
Physiological	Oxygen
Biochemical	Chlorpromazine
	Phenoxybenzamine
	Flunarizine
	Naloxone
	Centrophenoxine
	Etomidate

3. Classes of cyanide antidotes

A wide variety of compounds have been used as cyanide antidotes [9] and they can be broadly classified into four groups based on their putative mechanism of antagonism (Table 2). Sulfane sulfur compounds (prototype is sodium thiosulfate) are substrates for rhodanese (sulfurtransferase) which converts cyanide to thiocyanate. Scavengers are compounds that inactivate cyanide by binding it or by forming methemoglobin, which in turn binds cyanide. Oxygen appears to be a physiological antagonist which may facilitate dissocation of cyanide from cytochrome oxidase. The compounds classified as biochemical antidotes have largely unexplained mechanisms and their actions may be related to intracellular targets of cyanide other than cytochrome oxidase. Recent work suggests that the actions of many of these antidotes are not clear and may involve mechanisms different from those originally proposed [2]. These recent studies serve as the basis to develop new antidotes with increased efficacy.

4. Development of a mechanistic-based anti-cyanide screen

Since the CNS is extremely sensitive to cyanide, a number of neuronal processes can be

Table 3
Neurochemical markers of cyanide toxicity

Cytochrome oxidase inhibition
Activation of voltage sensitive Ca channels
Activation of receptor operated Ca channels
Elevation of cytosolic free Ca^{2+}
Activation of intracellular Ca cascades (PKC, PLA_2, PLC)
Inhibition of antioxidant enzymes (SOD, catalase, glutathione peroxidase)
Peroxidation of membrane lipids
Generation of ROS

used as neurochemical markers (Table 3). Detailed studies in a neuronal cell model (rat pheochromocytoma cell or PC12 cell) showed that six biochemical responses to cyanide could be used to establish a screen for evaluation of potential anticyanide compounds (Table 4). The goal was to screen for compounds that could inhibit the biochemical alterations caused by cyanide [10]. Compounds active in the screen could then be evaluated in animals for antidotal effectiveness. Potential antidotal activity was determined by computing a composite score in all six assays for each compound screened. To validate the assay, the rankings in the in vitro screen were correlated with their in vivo protective effects (LD_{50} values). Over 50 compounds or their combinations, including anticonvulsants, adrenergic blockers, antioxidants, and antipyschotics were tested. Based on the composite scoring in all six assays, carbamazepine, mannitol, allopurinol and phenytoin were ranked respectively as the most effective anti-cyanide compounds. The efficacy of these compounds remains to be fully evaluated in animal studies.

Table 4
Chemical markers of PC12 cells used in anticyanide multiple stage assay

Marker	Effect of cyanide
Cytochrome oxidase	Inhibits
Dopamine release	Stimulates
Cytosolic free Ca	Increases
Catalase	Inhibits
Superoxide dismutase	Inhibits
Intracellular ROS	Stimulates

5. Anti-oxidant defense and antidote development

Recent studies demonstrate that cyanide produces an immediate intracellular generation of reactive oxygen species (ROS) [6,11,12]. This response is summarized in Fig. 1. The process underlying this action of cyanide is unknown, but may result from mitochondrial dysfunction and/or activation of calcium sensitive processes. Compounding the stimulation of ROS production, the enzymes of the antioxidant defense (catalase, superoxide dismutase and glutathione peroxidase) are inhibited by cyanide. ROS can function as oxidative modulators of neuronal function and may account for some of the acute neurological actions of cyanide. On the other hand, it is well know that ROS can initiate cell injury and may play a role in the neuronal injury associated with subacute toxicity or the post intoxication sequela observed in some cases of acute cyanide intoxication. Based on these observations, catalase activity (the most sensitive of the antioxidant enzymes to cyanide) was used to screen for potential anticyanide compounds.

Known cyanide antidotes were screened for their ability to reverse cyanide inhibition of catalase in PC12 cells and this in vitro action was correlated with in vivo activity of known antidotes (LD_{50} values). The positive correlation indicates that protection of catalase activity can be used to screen for anti-cyanide activity. Preliminary results suggest anti-oxidants have a potential for antidote development and future work will focus on this class of compounds.

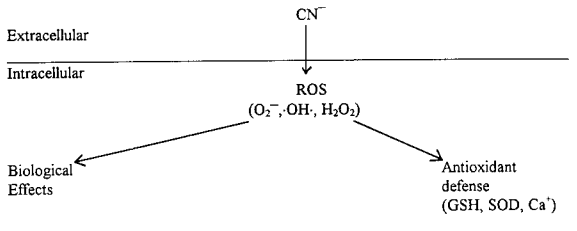


Fig. 1. Cyanide stimulates intracellular generation of reactive oxygen species (ROS) which in turn either activate normal intracellular processes or produce oxidative damage.

6. Nitric oxide generators as anti-cyanide compounds

Nitric oxide (NO) is a well characterized modulator of neuronal function. In the CNS, NO has been shown to modulate the NMDA glutamate receptor subtype by reacting with a redox site on the receptor [13]. Oxidation of the receptor redox site decreases opening of the receptor channel, thereby decreasing activity of glutamate. Glutamate is the most abundant neurotransmitter in the CNS and activates excitatory pathways. Recent studies have shown that cyanide activates the NMDA system and this may account for a number of the responses (seizures, neurodegeneration). It is possible that cyanide can reverse NOs actions on the receptor, resulting in an enhanced activation of the receptor. These mechanistic studies suggest NO may reverse cyanide actions on the glutamate NMDA system and it is reasonable to propose that NO generators may have antidotal activity.

Isosorbide dinitrate (ISDN) is a NO generator which was screened in mice for its ability to antagonize cyanide lethality [14]. ISDN alone (300 mg/kg, p.o.) elevated the LD_{50} of cyanide threefold, similar to that of sodium nitrite (100 mg/kg). This dose of ISDN did not elevate blood methemoglobin above control. To ensure that methemoglobin did not play a role in ISDNs antagonism, mice were pretreated with methylene blue, a compound which converts methemoglobin to hemoglobin. The antidotal activity of ISDN was not altered by the methylene blue treatment. On the other hand 100 mg/kg sodium nitrite produce 50% methemoglobin levels. Interestingly, by lowering the dose of sodium nitrite to 30 mg/kg, the methemoglobin levels were at control levels and the LD_{50} returned to that observed in mice receiving no antidotes.

These results show that ISDN is an effective cyanide antidote and its mechanisms of action are not the same as that of sodium nitrite. Since generation of NO is a well known action of ISDN as an antianginal agent, it is possible that NO generation is also the mechanism of cyanide antagonism by ISDN.

7. Conclusions

Based on these studies it is apparent that sensitive markers of toxicity can be identified from mechanistic studies which can be used in studying compounds for potential antidotal activity. By using mechanistic based antidote design, several new classes of lead compounds have been identified and additional animal testing is required to establish their antidotal efficacy.

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Toxicology Letters

Drug-specific antibodies as antidotes for tricyclic antidepressant overdose

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Abstract

Drug-specific antibodies have been used clinically to treat digoxin or colchicine overdose. The lethal dose of tricyclic antidepressants (TCAs) is 100 times higher, and will require higher doses of antibodies (up to several g/kg) to reverse toxicity. Preliminary studies suggest that this is feasible. High affinity TCA-specific monoclonal Fab′ or polyclonal Fab fragments rapidly reverse the cardiovascular toxicity of the TCA desipramine (DMI) in rats, and prolong survival. TCA-specific Fab′ or Fab is generally well tolerated in rats, but doses several times higher than anticipated for human use may have adverse effects. Combining Fab with standard therapies for TCA overdose, such as $NaHCO_3$, can reduce the required Fab dose. As an alternative, a recombinant single chain Fv fragment (sFv), one half the size of Fab, has been cloned which retains a high affinity for DMI and is able to alter DMI distribution in vivo. Because sFv has a shorter elimination half-life and more extensive renal excretion than Fab, it may have therapeutic advantages.

Keywords: Tricyclic antidepressant; Drug-specific antibody; Immunotherapy

Drug-specific antibodies represent an attractive approach to the treatment of drug overdose. Protein (or antibody) bound drug is generally inactive. Administration of a sufficient dose of drug-specific antibody can bind drug, and prevent its distribution to target tissues or redistribute it out of target tissues after distribution to these sites has taken place [1]. If the 50-kDa Fab fragment is used rather than intact IgG (Fig. 1), this fragment is largely excreted in urine, providing a route of elimination to both Fab and Fab-bound drug [2]. Because high affinity antibodies can be readily produced for most drugs, this strategy is potentially applicable to virtually any type of drug overdose.

The clinical usefulness of drug-specific Fab has been well established for digoxin overdose. Administered at an equimolar dose, digoxin-specific Fab reverses digoxin toxicity and is well tolerated [3]. More recently, a drug-specific Fab has also been used to reverse toxicity in a patient with colchicine overdose [4]. These applications demonstrate the feasibility of this therapy and provide interventions for two overdoses that are

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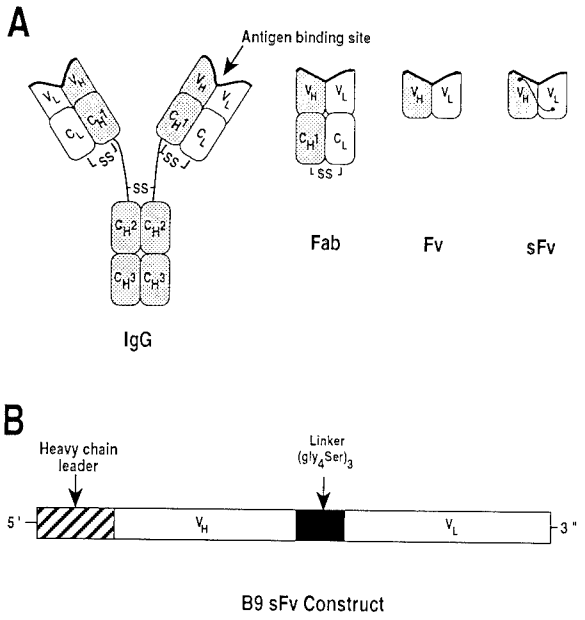


Fig. 1. Schematic representation of antibody and antibody fragment structures. Single chain Fv fragment is composed of V_H and V_L regions joined by a flexible 15 amino acid linker that tethers these regions together but does not affect hapten binding. Adapted with permission from Huston et al. [16].

otherwise very difficult to treat. However, both digoxin and colchicine are atypical drugs in that they are very potent; their toxic dose is quite low and the dose of Fab fragment required to reverse toxicity is correspondingly low. Most drugs of clinical interest have toxic doses 10–100 times higher and will require proportionally higher doses of Fab to treat overdose. For example, drugs such as tricyclic antidepressants, barbiturates, aspirin, and theophylline all have toxic doses that are much higher than that of digoxin or colchicine (Table 1). The generality of drug-specific Fab as an antidote depends entirely upon whether this therapy can be scaled up by several orders of magnitude.

Tricyclic antidepressant (TCA) toxicity is the leading cause of death from intentional drug overdose in the USA [5]. The toxic dose of the TCAs is more than 100 times that of digoxin. TCA toxicity therefore provides a reasonable and clinically important model for studying the feasibility of drug-specific Fab for drugs that have a very high toxic dose. General questions that must be addressed include (1) are the pharmacokinetic properties of Fab (small volume of distribution, rapid renal elimination) altered at high doses, (2) is therapeutic efficacy maintained at high Fab and drug doses, (3) is high dose Fab in itself toxic, and (4) is high dose Fab production economically feasible. Preliminary studies have begun to address these issues.

The pharmacokinetic properties of digoxin-specific Fab that allow its efficacy are a small volume of distribution (approximating the extracellular space), a short half life (24 h in humans), and substantial renal elimination (>50% of total clearance) [6]. Pharmacokinetic studies of high doses of Fab have been performed in rats and dogs using nonspecific human Fab. In rats receiving a Fab dose of 7.5 g/kg, pharmacokinetic parameters (Table 2) closely approximated those reported for lower doses of digoxin-specific Fab in dogs, baboons, or humans

Table 1
Toxic doses of some drugs and the equimolar dose of Fab

Drug	Toxic dose (mg)	Fab dose (g)
Digoxin	15	1
Colchicine	80	10
Tricyclic antidepressant	2000	333

Table 2
Comparison of pharmacokinetic parameters of high doses of nonspecific Fab in rats and dogs[a]

	V_{ss} (L/kg)	Cl_T (ml/kg/h)	Cl_R (ml/kg/h)	F_{urine}	β t/2 (hours)
Rat	0.43	27.2	8.9	0.31	16.3
Dog	0.43	9.2	1.9	0.10	44.5

V_{ss}, steady state volume of distribution; Cl_T, total body clearance; Cl_R, renal clearance; F_{urine}, fraction excreted in urine; β t/2, terminal half-life.

[a]Fab dose: rats 7.5 g/kg i.v. over 1 h, dogs 5.2 or 3.2 g/kg (data combined) i.v. over 1 h.

[7]. The elimination half-life of high dose nonspecific Fab in dogs was somewhat longer and the fraction excreted in urine somewhat lower, but still in a range that should allow Fab to be an effective antidote [8]. Thus the pharmacokinetic properties of Fab appear to be similar over a wide range of Fab doses, including those that would be needed to treat TCA overdose.

High doses of Fab were well tolerated in rats, with no changes in cardiovascular parameters acutely, normal wakening from anesthesia, and normal organ histology at 21 days [7]. Dogs also tolerated the Fab infusion well, but developed transient renal insufficiency [8]. Whether this was due to the Fab or to contaminant proteins is unclear. All animals survived for 21 days and had normal organ histology (including kidneys) at that time. Thus administration of high doses of Fab fragment appears possible, but more safety data are needed.

The efficacy of DMI-specific antibody fragments has been demonstrated in rats with DMI toxicity by their ability to redistribute DMI from tissues into serum, enhance DMI renal excretion, reduce cardiovascular toxicity, and prolong survival [2,9–11]. DMI toxicity in rats (and humans) is characterized by hypotension and prolongation of the electrocardiographic QRS interval [12]. Several different DMI-specific antibodies have been studied with regard to their effects on these parameters. A high affinity ($K_a = 3 \times 10^8$ M^{-1}) monoclonal antibody was studied after its digestion to the 55-kDa Fab′ fragment, which is about 10% larger than Fab but appears to have similar pharmacokinetic properties (the Fab′ fragment was used because it is easier to produce in large quantities from this particular monoclonal antibody) [9]. Rats received DMI 30 mg/kg i.p. followed by 1 g/kg of G5-Fab′, a molar Fab′/DMI ratio of 0.09. G5-Fab′ rapidly increased the DMI serum concentration 15-fold, demonstrating the redistribution of DMI out of tissues. Concurrently, the G5-Fab′ reduced QRS prolongation, illustrating several important points. First, therapeutic effect was achieved rapidly, with onset during the 10-min Fab′ infusion and maximal effect by the end of infusion (Fig. 2). This is critically important for TCA overdose, as most

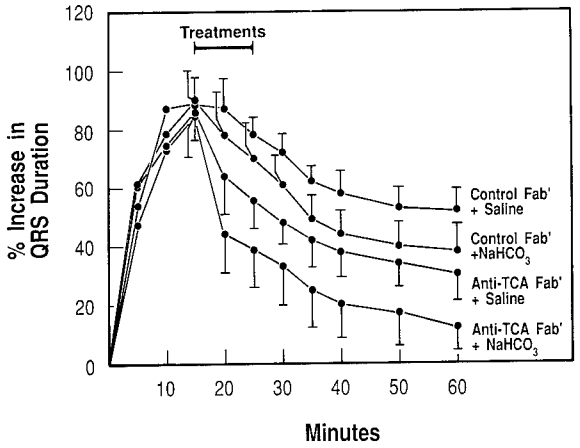


Fig. 2. Effects of G5 (anti-TCA) Fab′ fragment and hypertonic $NaHCO_3$ on the percent change in QRS duration. DMI 30 mg/kg was administered at 0 min, and treatments were administered starting at 15 min. DMI prolonged QRS duration, and anti-TCA Fab′ alone produced a rapid reduction in QRS duration ($P < 0.01$). The combined effect of anti-TCA Fab′ and $NaHCO_3$ was greater than that of either treatment alone ($P < 0.001$). Reprinted with permission from Brunn et al. [9].

deaths occur in the first 1–2 h after ingestion and rapid treatment is essential. Second, G5-Fab′ efficacy was observed at a relatively low Fab′/DMI molar ratio, unlike the equimolar ratio usually used to treat digoxin overdose. Whether this is due to DMI having a steep dose-response curve, or the selective redistribution of DMI from target sites is not clear. In either case, the unexpectedly low Fab′ dose required should be helpful in minimizing both the possibility of toxic effects from the antibody and its cost. G5-Fab′ is also effective in improving blood pressure in rats with DMI toxicity, again at a relatively low molar ratio (Fig. 3) [9].

Another strategy to reduce the required Fab′ dose is to combine it with other treatments. Hypertonic $NaHCO_3$ has been shown to reduce the cardiotoxicity of TCAs, although it is often ineffective in the most seriously poisoned patients. $NaHCO_3$ acts as a physiologic antagonist, reducing the TCA-induced sodium channel blockade that is responsible for TCA cardiotoxicity [13]. The combined use of $NaHCO_3$ and DMI-specific Fab′ produces a greater improvement in

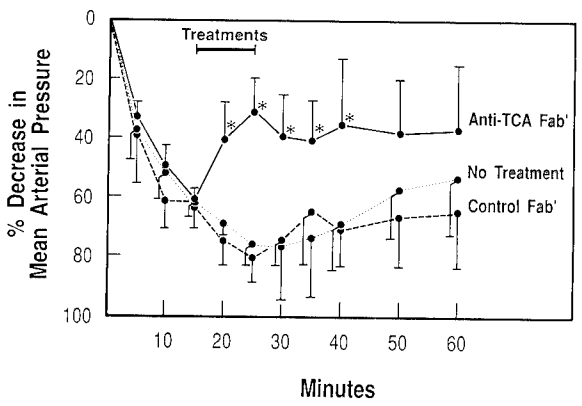


Fig. 3. Effects of G5 (anti-TCA) Fab′ fragment on hypotension. DMI 60 mg/kg was administered at 0 min, and treatments were administered starting at 15 min. DMI produced substantial hypotension, and anti-TCA Fab′ rapidly improved blood pressure ($P < 0.01$). Reprinted with permission from Brunn et al. [9].

DMI-induced QRS prolongation than either treatment alone [9]. Presumably this beneficial interaction results from combining pharmacodynamic ($NaHCO_3$) and pharmacokinetic (Fab) antidotes.

The G5 monoclonal antibody has been useful in establishing the efficacy of this approach to DMI overdose in the rat model. However, polyclonal antibodies may have advantages as a pharmaceutical agent for several reasons. First, polyclonal antibodies typically have higher affinities for a given hapten than their monoclonal counterparts. Second, it is possible to produce polyclonal antibodies with broad specificity, an important advantage for TCA overdose in that more than a dozen different TCAs are in use worldwide. Third, polyclonal antibodies may be less expensive to produce if high titers are achievable. We therefore evaluated a polyspecific, polyclonal affinity-purified Fab as a treatment for DMI toxicity [14]. This Fab (designated TFab) has a very high affinity for DMI (1.4×10^{10} M^{-1}), but also has high affinities for imipramine, amitriptyline, and nortriptyline. As with monoclonal Fab′, TFab rapidly redistributes DMI and reverses DMI-induced QRS prolongation in rats. Doses of 1 or 2 g/kg (TFab/DMI ratios of 0.11 or 0.22) are effective. The lower cost of this polyclonal Fab has allowed the use of larger doses to treat rats receiving higher (fatal) doses of DMI [9]. When DMI is infused continuously until death, TFab at a dose of 2 g/kg (molar Fab/DMI ratio 0.21) prolonged survival by 50% (Table 3). This relatively low molar ratio of Fab/DMI is therefore effective not just for surrogate markers of outcome (QRS duration, hypotension), but also for prolonging survival. These data support the further study of drug-specific Fab as a clinical treatment of TCA overdose.

Although highly effective as antidotes, some caution is warranted with regard to the potential side effects of high dose Fab therapy. In studies of TFab in rats, several rats died after initial improvement [14]. TFab is not in itself toxic, as doses higher than anticipated for clinical use (4 g/kg) are well tolerated in anesthetized rats in the absence of DMI [15]. Possibly the combined effects of DMI toxicity, several hours of anesthesia and the large protein and volume load associated with antibody administration caused the observed deaths. The previously noted ability of TFab to prolong survival in rats during DMI infusion argues that TFab is nevertheless beneficial in seriously poisoned animals. Clearly, efforts to reduce the required TFab dose, such as concurrent administration of $NaHCO_3$, should be pursued.

Another strategy for reducing the antibody dose is the use of still smaller fragments. The protein domains responsible for hapten binding are contained within the N-terminal half of the Fab fragment. It is possible to produce a 25-kDa Fv fragment that fully retains the ability of the

Table 3
Effects of a TCA-specific polyclonal Fab (TFab) on survival in rats receiving a constant infusion of DMI until death. Duration of survival and cumulative DMI dose (mean ± S.D.) were both substantially increased by TFab administration ($P < 0.01$) compared to control groups that received NaCl or albumin

Group	Time of death (min)	DMI dose (mg/kg)
NaCl	31.8 ± 6.3	63.5 ± 12.6
Albumin	31.6 ± 6.2	63.3 ± 12.5
TFab	50.0 ± 8.5	100 ± 16.9

parent IgG to bind hapten (Fig. 1). However, this fragment is unstable because it lacks the covalent interchain disulfide bond of the Fab fragment, and is therefore difficult to produce. As an alternative, it is possible to clone the V_H and V_L genes, introduce a linker sequence, and express a recombinant single chain Fv fragment with V_H and V_L chains covalently joined by a 15 amino acid peptide that acts as a tether and stabilizes the protein [16]. Such single chain Fv fragments (sFv) can be expressed at high levels in cultured cells. Potential advantages of recombinant sFv as an antidote compared to Fab are its smaller size (and therefore dose), more extensive renal excretion, and the potential to engineer the fragment to alter and improve its therapeutic properties [17,18].

DMI-specific sFv has been cloned from a monoclonal antibody (designated B9). The B9-sFv can be expressed at very high levels (up to 40 mg/l) in cultured NS0 myeloma cells and purified in one step by direct application of medium to an affinity column. This fragment has nearly the same affinity for DMI as the corresponding Fab (1.3 vs. 2.0×10^8 M^{-1}). Preliminary studies with small doses of B9-sFv show that it can alter DMI pharmacokinetics by rapidly redistributing DMI from tissues into serum (unpublished data). The extent of redistribution was greater for B9-IgG or B9-Fab, but these also have smaller volumes of distribution than sFv and, consequently, higher serum concentrations. Thus the lesser redistribution of DMI after B9-sFv may reflect its more rapid distribution out of serum rather than lesser efficacy. These data support the study of recombinant sFv as an alternative to monoclonal or polyclonal antibody fragments. A potential disadvantage of B9-sFv (and of sFv in general) is its poor stability at 37°C. Site directed mutagenesis to improve stability has been successful for some sFv fragments [19,20] and demonstrates the advantage of having a recombinant protein that can be engineered to improve its therapeutic properties (or reduce toxicity if this proves to be a problem). Further studies of B9-sFv to determine whether it reverses DMI toxicity are clearly of interest.

In summary, the use of drug-specific antibodies may represent a general strategy applicable to the treatment of a wide variety of drug or chemical toxicities. To accomplish this, it is necessary to scale up this intervention several orders of magnitude from its two demonstrated clinical applications, digoxin and colchicine overdose. Animal studies of DMI overdose suggest that this is feasible, but the high Fab dose required poses challenges with regard to both safety and cost. The use of strategies to minimize the Fab dose, or the use of smaller recombinant antibody fragments, offer possible means of overcoming these difficulties.

Acknowledgements

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Genetically engineered bacterial cells and applications

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Abstract

Heterologous expression systems have proven to be very important in P450 research, permitting investigation of many P450s identified only by recombinant (cloning) technologies. They also make it possible to study human P450s since tissue sources for naturally occurring enzymes are difficult to obtain. A variety of different heterologous systems have been applied to the study of P450s and each one has its own unique advantages. For the study of biophysical properties and structure/function relationships, *E. coli* has proven to be a particularly good system. Both microsomal and mitochondrial P450s can be expressed to high levels in *E. coli* and subsequently purified to homogeneity for detailed analysis. Techniques of both site directed mutagenesis and random chimeragenesis are very facile in bacteria providing excellent opportunity to analyze specific aspects of structure/function relationships of P450s. Furthermore microsomal P450s are active in intact *E. coli*, activities being supported by flavodoxin and flavodoxin reductase, providing the opportunity to develop bioreactors expressing designer P450s. The salient features of P450 expression in bacteria are summarized herein.

Keywords: P450 expression; *E. coli*; Flavodoxin; Chimeragenesis; P450scc; P450c17

1. Introduction

The remarkable development of the techniques of molecular biology has made possible the cloning of a great many new forms of P450, including previously inaccessible human forms. This technology has led to the discovery of many P450s having unknown enzymatic properties. To investigate the catalytic activities of these newly discovered enzymes, the use of heterologous expression systems has been applied to the study of P450s. The reasons for developing heterologous P450 expression systems are several, including:

1. Opportunity to study activities of specific forms of P450 in the absence of other forms.
2. Opportunity to study the capacity of a specific form of P450 to metabolize a lead compound and to obtain sufficient amounts of resulting metabolites for structural analysis.
3. Large-scale production of P450s for structural and other biophysical studies.
4. Generation of bioreactors containing P450 activities to be used in commercial processes for chemical synthesis and bioremediation.

A variety of different heterologous expression

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systems have been shown to be useful in P450 research including, yeast, COS cells, vaccinia virus, transformed animal cell lines, baculovirus and *E. coli* [1]. Each of these systems has its own unique properties and will be selected for use by different laboratories depending on their particular needs. Two of these systems are of particular use for large scale production of P450s: baculovirus (Sf9 cells) and *E. coli*. The particular reasons for wanting to produce large quantities of P450s revolve around the study of structure/function relationships. Of course, three-dimensional structure determination requires large quantities of highly purified protein. For the study of most eukaryotic P450s, particularly human forms, overexpression in a heterologous system is the only practical way to obtain sufficient quantities for crystallography. Furthermore, to translate structural information into understanding of P450 function, it is necessary to be able to modify the P450 sequence and to measure in detail the various properties of the modified enzyme. Both baculovirus and *E. coli* are suitable for these needs. We have compared expression levels of the same P450, bovine 17α-hydroxylase cytochrome P450, (P450c17) in different expression systems [2] and have found that *E. coli* produces the largest amount of functional P450 per liter culture. Furthermore *E. coli* is much more facile for mutagenesis studies than is baculovirus and culture costs are significantly cheaper. This article focuses on the development of genetically engineered bacteria which overexpress eukaryotic P450s.

2. Expression vectors

All eukaryotic P450s are known to be integral membrane proteins. The majority of such P450s are localized in the endoplasmic reticulum and participate in an electron transport system consisting of:

NADPH → NADPH-cytochrome P450 reductase → P450
(FAD, FMN)

Perhaps as many as 10% of the eukaryotic P450s are localized to the inner mitochondrial membrane and participate in an electron transport system consisting of:

NADPH → Ferredoxin reductase → Ferredoxin → P450
(FAD) (2Fe-2S)

Utilizing different expression vectors we have developed overexpression systems for both microsomal and mitochondrial P450s. The pCWori$^+$ vector [3] has been found to be excellent for overexpression of microsomal P450s [4]. Other vectors have worked well for low expression of microsomal P450 [5,6] but based on our studies [2,3] and those of others [7–13], pCW is very useful for high level expression of many different microsomal P450s. Expression levels higher than 1.0 μmol/l culture have been achieved. The pTrc99A vector has served well for overexpression of the mitochondrial P450, cholesterol side chain cleavage cytochrome P450 (P450scc) [14] and recently has been found useful in another laboratory for expression of cholesterol 27-hydroxylase cytochrome P450 (P450c27) [15]. We have achieved levels of 250 nmol P450scc/l culture.

Requirements for expression of proteins in *E. coli* have been examined in detail. One of the key requirements is the second codon [16]. Certain triplets are much more effective as the second codon than others, and we have modified the amino terminal sequence of both the P450c17 and P450scc cDNAs so that the second codon is GCT(Ala) in the pCW construct [4] and GTC(Val) in the pTrc99A construct [14]. Furthermore, in the pCW construct (microsomal P450) silent changes were made in the first eight codons to enhance the A-T richness of this region to reduce the tendency for secondary structure formation of hairpin loops in the resultant RNA. The changes noted below (indicated in bold) led to the difference between no expression in *E. coli* strain JM109 as measured by immunoblot analysis (native sequence) and an expression level of 750 nmol P450c17/l culture (modified sequence) [4].

Native bovine P450c17:

ATG TGG CTG CTC CTG GCT GTC TTT
Met Trp Leu Leu Leu Ala Val Phe

Modified P450c17:

ATG**GCT**CTG**TTA**TTA**GCA**G**TT**TTT
Met Ala Leu Leu Leu Ala Val Phe

Several laboratories have found that by using the precise modified eight codons shown above at the 5′-end of the cDNA encoding their favorite P450, high level expression of forms from different CYP gene families can be obtained. Thus these eight codons are particularly favorable for expression of functional forms of microsomal P450s.

Having expressed bovine P450c17 to a high level in *E. coli* we next tried expression of human P450c17 following the same rules (in bold), but not the precise 5′ coding sequence, used for the bovine enzyme [17].

Native human P450c17:

ATG TGG GAG CTC GTG GCT CTC TTG
Met Trp Glu Leu Val Ala Leu Leu

Modified: ATG **GCT** GA**A** **TTA** **TTA** GC**A** CG**T** TTG
Met Ala Glu Leu Leu Ala Leu Leu

These modifications lead to production of only about 40 nmol P450c17/l culture, whereas the precise modified bovine sequence shown above when used in place of the first eight codons of human P450c17 leads to 400 nmol P450c17/l culture. This result further emphasizes how well the modified sequence used for expression of bovine P450c17 can be applied to expression of other microsomal P450s. Another interesting point from comparison of expression of bovine and human P450c17 is that even when using precisely the same first eight codons, bovine P450c17 is always expressed at about twice the level achieved for human P450c17. Thus the level of expression is also influenced by the remainder of the coding sequence; that of human P450c17 being less effective than that of bovine P450c17 for protein production in *E. coli*.

3. *E. coli* as a bioreactor

One of the futuristic yet practical goals of P450 research is to be able to develop systems which take advantage of the unique chemistry catalyzed by P450s to carry out important commercial tasks. For example, the use of P450s can be imagined in synthesis of drugs and fine chemicals, use in pollution control and bioremediation, and perhaps even for the development of biomedical replacement systems. One strategy for this goal is to develop microorganisms which can carry out such tasks. For example, yeast which express a complement of CYP genes and contain an endogenous microsomal NADPH-cytochrome P450 reductase, could serve such a role following transformation with cDNAs encoding recombinant P450s.

A surprising result from the development of the expression system for bovine P450c17 in *E. coli* was that the enzyme was active in intact bacteria [4]. This was surprising for two reasons. First, mitochondria are thought to have evolved from bacteria and one might have predicted that mitochondrial P450s would be active in *E. coli* (they are not) rather than microsomal P450s. Second, *E. coli* are not known to express P450s and are reported not to contain NADPH-cytochrome P450 reductase [18]. The *E. coli* 'P450 reductase system' was found to be in the cytosol, not the membrane fraction which contains the P450c17 [4]. We have purified this P450 reductase system finding it to be composed of two components, flavodoxin and flavodoxin reductase [19]. Flavodoxin is a 19-kDa, FMN-containing protein and flavodoxin reductase is a 29-kDa FAD-containing, NADPH-binding protein. Thus the electron transport system is:

NADPH → Flavodoxin reductase → Flavodoxin → P450
(FAD) (FMN)

It is possible that NADPH-cytochrome P450 reductase evolved from this two component system [18]. Presence of flavodoxin/flavodoxin reductase which supports microsomal p450 activities in *E. coli* suggests that this microorganism could be quite useful as a bioreactor following transformation of recombinant microsomal P450. The activity produced by the endogenous flavodoxin/flavodoxin reductase system is about 40% that obtained in a reconstitution system using purified rat liver P450 reductase. Thus overexpression of both flavodoxin and flavodoxin re-

ductase might be necessary to develop an efficient bioreactor.

Another approach to generating such a bioreactor is derived from the characterization of the bacterial P450, $P450_{BM-3}$. $P450_{BM-3}$ is a fusion protein consisting of an N-terminal P450 domain and a C-terminal P450 reductase domain [20]. This enzyme has a very high turnover number suggesting that expression of a recombinant fusion protein might lead to elevated P450 turnover numbers in microorganisms. This strategy was shown to work well in yeast [21] and has now been applied to *E. coli* [8]. These fusion proteins with moderately elevated turnover numbers will permit development of bioreactors having P450 activities similar to those measured in animal cells. Further enhancement of the enzymatic activity of these fusion proteins or overexpression of flavodoxin/flavodoxin reductase in order to produce microorganisms having high levels of P450 activity remains to be accomplished. However, the unique chemistry catalyzed by P450s and the potential for engineering P450s to catalyze very specific reactions, makes development of such bioreactors having high level P450 activities an important goal.

4. *E. coli* and P450 structure/function

Not only can P450s (both mitochondrial and microsomal forms) be overexpressed to high levels in *E. coli*, they can be purified to homogeneity from bacterial membranes. Modification of standard chromatography protocols for purification of P450s from animal cells has proven very successful [22,23]. In addition, because of the facile nature of recombinant DNA technology, it has been possible to produce a functional human P450c17 having four histidine residues at the C-terminus. These histidines serve as an affinity tag for purification of the P450 on a Ni^{+2}-affinity column. Other purification protocols will surely be developed in time, but the key point is that when using expression in *E. coli* it is certainly possible to generate large quantities of highly purified, functional P450 enzymes. This protein is suitable for crystallization attempts. We are presently trying to produce crystals of bovine P450scc which can be used for structure determination. This protein has previously been crystallized after modification by 5′-pyridoxal phosphate, but these crystals have not proved useful for structure determination [24]. At this writing we have invested 200 mg of highly purified, recombinant bovine P450scc from *E. coli* in crystallization efforts, testing different precipitants, detergents, and effectors. At this stage we have no crystals but we do have a crystalline precipitate. Patience and luck will be necessary for elucidation of tertiary structure of eukaryotic P450s. However, the development of high level *E. coli* expression systems for most P450s makes such attempts reasonable.

Even when we determine the high resolution structure of P450scc, we will still not fully understand how the enzyme works. As has been shown so elegantly for hemoglobin, combination of detailed structural information with functional data obtained from mutant proteins can lead to a very detailed understanding of how a protein functions. One of the other assets of P450 expression in *E. coli* is the facility with which mutagenesis can be carried out. We have used this approach to identify the adrenodoxin binding site on P450scc [22] and to investigate the active site [25], both by site-directed mutagenesis. However, it is not always possible to identify exactly which amino acid should be modified in order to localize residues associated with a specific property of a particular P450. We have undertaken application of the novel approach of random chimeragenesis in *E. coli* to investigate P450 structure/function. This approach can be applied to expression in either yeast or *E. coli*. Briefly stated, two cDNAs are placed in tandem in an expression plasmid. This vector is linearized with restriction endonucleases and the linearized DNA is transformed into *E. coli*. Recombination occurs between the two cDNAs producing functional chimeric proteins. Pompon and colleagues have utilized this approach in yeast to study the closely related P4501A1/P4501A2 system [26]. We have taken a different approach, rather than focusing on sequence relatedness, by focusing on enzymatic activity.

As seen in Fig. 1 we have generated chimeras between P450scc and P450c27. Even though these two enzymes are members of different P450 gene families, having only about 25% sequence identity, they metabolize the same substrate, cholesterol. P450scc hydroxylates the cholesterol side chain at c20 and c22 leading to cleavage by a third O_2 dependent step to produce pregnenolone. P450c27 hydroxylates the side chain at c27. Construction of chimeras between these two enzymes should lead to localization of regions within the primary sequence which participate in side chain hydroxylation at different positions. The one chimera which has been examined in preliminary studies, has an N-terminal P450scc sequence and at amino acid 107 continues on as P450c27. Thus it contains substrate recognition sequence 1 of P450scc and the remaining 5 substrate recognition sequences from P450c27 [27]. The enzyme can catalyze production of pregnenolone from cholesterol at about 15% the level of wild type P450scc. We have not yet examined its 27-hydroxylase activity. However, it is clear from these preliminary data that random chimeragenesis can be applied to study sequence requirements for specific P450 activities and will lead to new insights into P450 structure/function relationships.

Thus *E. coli* expression has proven to be very useful for the study of P450 structure/function. First, it permits large scale production of P450s for biophysical studies. Second, it is a very facile system for both site-directed mutagenesis and chimera production. *E. coli* is particularly important for this latter point because purification of mutant or chimeric enzymes is easily achieved.

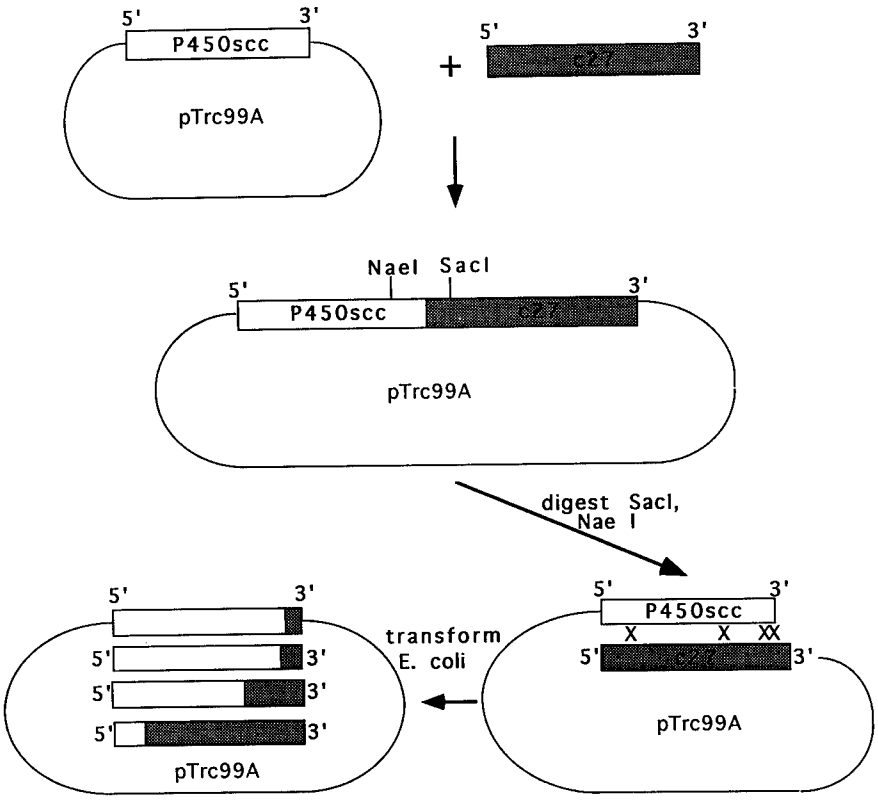


Fig. 1. Schematic of the protocol for generation of linearized pTrc99A containing cDNAs encoding bovine P450scc and human P450c27, used for random chimeragenesis. Sites where crossovers were obtained are indicated by X.

Table 1
Expression of bovine P-450c17 in different expression systems: relative levels determined by immunoblot analysis from 25 μg membrane protein

Expression system	Relative level (%)
Bovine adrenal microsomes	100
COS microsomes	25
Yeast microsomes	80
Sf9 (insect) microsomes	200
E. coli membranes	300

Detailed analysis of the functional properties of such modified enzymes is best carried out with purified proteins.

5. Conclusions and future directions

The development of *E. coli* expression systems for the study of P450s has proven to be an important addition to this research area. High level expression compared to other widely used expression systems (Table 1) has important advantages. In particular this system is useful for providing key reagents for study of P450 structure/function relationships. Second, this system has potential as a bioreactor containing specific P450 activities. We can imagine that not too far into the future, designer P450s which catalyze activities required for specific enzymatic tasks, will be available in *E. coli* bioreactors on a commercial basis.

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Genetically engineered yeast cells and their applications

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Abstract

The first generation of yeast expression systems relies on inducible expression cassettes borne by multicopy plasmids for production of unmodified human P450s and on the endogenous NADPH-P450 reductase to support activities. A second generation of engineered yeast involved targeted genomic modifications allowing overexpression of the yeast reductase and coexpression of human cytochrome b_5 and of a phase II enzyme such as epoxide hydrolase. These features allow improved P450 turnover numbers and simulation of some phase I-phase II couplings. In the third generation, the human reductase was substituted for the yeast reductase by genome engineering. Simultaneously, induction procedures were optimized to reach high P450 specific contents. Dramatic improvements (1000-fold) of yeast-expressed P450 activities have thus been obtained. To get more insight into complex metabolic events, such as that of a typical pollutant: benzo[*a*]pyrene, an approach was designed which involves a complementary use of yeast expression and computer simulations.

Keywords: Yeast heterologous expression; Simulation; Human P450 and epoxide hydrolase

1. Introduction

The development of molecular biology techniques has led to an exploding increase in the number of known human P450 and phase II sequences. Ascribing a precise substrate specificity to newly cloned P450 sequences became a critical task making the availability of heterologous expression systems crucial. Oeda et al. reported in 1985 the first expression of a cDNA encoding a mammalian microsomal cytochrome P450 in *Saccharomyces cerevisiae* [1]. The recombinant P450 1A1 was found properly incorporated into the yeast endoplasmic reticulum and the activity supported by coupling with the endogenous yeast NADPH-P450 reductase (reductase). Following this first report, expression of a large number of P450-encoding cDNAs in *S. cerevisiae* was reported in parallel with the development of other heterologous expression systems involving alternative hosts such as *E. coli*, insect and mammalian cells [2,3]. Among the relative advantages of each system, bacteria-based expression offers frequently a high yield but requires some N-terminal sequence alterations of the P450s in order to be expressed. This system is generally not self-sufficient for activity

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analysis since reductase and cytochrome b_5 are absent from the bacteria. In contrast, expressions in mammalian cells as COS1, V79 or human B lymphoblastoid cell lines [3,4], gave rise to systems well suited for direct toxicological or metabolic studies [5,6], but frequently at a low P450 yield and high cost.

The first developed yeast expression systems featured easy and low cost P450 expression, but enzyme content and specific activity remained rather low. The reason for the low specific activity was the low level of endogenous (yeast) reductase present in wild type cells and in some cases the poor recognition between endogenous reductase and heterologous P450 [7,8]. Surprisingly, although yeast contains significant amounts (about 30–100% of the expressed P450 level) of endogenous cytochrome b_5, it is unable to enhance human P450 activities as does the rodent equivalent [9]. In addition most of human liver phase II activities are absent from yeast cytoplasm. These observations led us to alleviate these difficulties by considerably improving the P450 yield per volume of culture and by the development of genetically engineered strains that can provide an optimised human redox environment for P450 function and when required some phase II activities [10,11].

2. The 3 generations of yeast expression systems

2.1. Optimising the P450 yield

The first step toward optimisation of the yeast expression system was to improve as much as possible the P450 specific contents. The production in *S. cerevisiae* of heterologous P450 is based on the construction of expression cassettes containing the coding phase of the considered P450 cDNA sandwiched between yeast transcription promoter and terminator sequences. A first optimisation criterion is the deletion of the cDNA 5′-non-coding sequences before insertion of the open reading frame (ORF) in the yeast expression vector. Cloning and formatting are performed in a single polymerase chain reaction amplification step using total cDNAs as template and a thermostable polymerase with a proof-reading activity feature. A second optimisation was to systematically use an inducible promoter for expression. The rationale for this being to separate the exponential growth phase required to constitute the biomass from the expression phase, for which a nearly stationary biomass condition allows an improved accumulation of heterologous protein. In addition, such a phase separation avoids the presence of a negative selection against high plasmid copy numbers during cell division. Induction by a change in the carbon source from glucose to galactose is routinely used in the laboratory. Nevertheless, an intermediate growth phase in ethanol is required before induction to facilitate the transition to galactose utilisation under high density conditions. P450s produced in *S. cerevisiae* cells are now obtained in high yield in the natural folded state without any N-terminal sequence alteration, as is required for bacterial expression [3]. Depending on the P450 isoform, microsomal P450 contents are in the range of 50–400 pmol (average of 200 pmol) of spectrally detectable P450 per mg protein. Owing to the high culture densities that can be reached with yeast, production of several hundred nmol of an individual P450 per l culture is routinely achieved. This level is more than sufficient to use microsomal fractions as such for spectral analysis such as substrate/inhibitor binding analysis, without the need for purification since no endogenous P450 is spectrally interfering.

2.2. First optimisation of P450 specific activities

Yeast cells exhibit a moderate level of endogenous P450 reductase activity (about 100 nmol cytochrome *c* reduced per min and per mg microsomal protein). Therefore, the basic yeast system is partially self-sufficient. Nevertheless, the observed P450 turnover numbers are often much lower than those determined in vitro after purification and reconstitution with an excess of mammalian P450 reductase. Simple experiments clearly demonstrated that the reductase level is the limiting factor [7], while the molar ratio of reductase to P450 is in the range of 0.05–0.5, a value which compares well with ratios observed in human liver microsomes. This indicates that

not only the limited amounts of endogenous reductase, but also the heterologous nature of interactions with P450, could be the cause of observed limitations. Two solutions were thus considered: one is to strongly increase the level of endogenous reductase in order to compensate its lower efficiency; the second is to substitute the yeast reductase by the human enzyme. The first solution involved a genetic engineering of the yeast reductase locus leading to the W(R) strain series, which was widely analysed and reviewed in previous papers [9–11]. The strong yeast reductase overexpression achieved (about 25-fold the wild type level) was found useful to widely improve (5–70-fold) most of P450 activities expressed in yeast.

A second important point is the role of cytochrome b_5. This cytochrome is an alternate electron carrier for the second electron transfer involved in the catalytic cycle. The cytochrome b_5-dependent improvement in P450 activity is strongly related to the P450 isoenzyme considered, on the type of substrate oxidised, and, most importantly, on the concentration and nature of the P450 reductase present. Thus, in specific situations the highest P450 activity can be achieved either by high levels of yeast reductase only or by suitable association of lower reductase levels and cytochrome b_5. This led us to build a series of genetically engineered yeast strains (Fig. 1) associating variable expression levels of yeast reductase and human cytochrome b_5 with a plasmid-borne P450 expression cassette (see [10] for review).

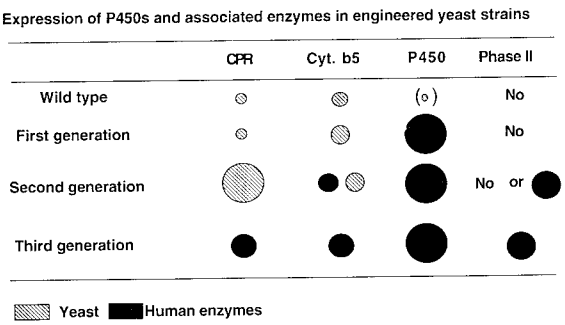


Fig. 1. The 3 generations of yeast expression systems. Disk size is indicative of the relative expression levels. See text and references for the corresponding strain constructions.

2.3. Shifting from yeast P450 reductase to the human enzyme

The described systems are rather satisfying when the aim is to analyse qualitatively human P450 activities. Nevertheless, the strong dependency of P450 activities toward their redox environment and the observation that changes do not necessarily modify all activities in the same way raised the question of the fidelity of the quantitative simulation of human liver metabolism by the heterologous system. From this point of view the best system is not necessarily the more active. For example, the very high level of reductase in a yeast strain like W(R) is of clear importance to enhance human P450 1A1-dependent metabolite formation, but can lead to an overestimation of the contribution of this P450 in vivo, where much lower reductase levels are found (similar contents of human and yeast reductases are found to be of similar efficiency). In contrast, human P450 3A4 requires high levels of yeast reductase to compensate the rather poor efficiency of its coupling with the yeast enzyme in comparison to the human one. These findings led us to consider an alternate strategy where the highest P450 specific activity is not the goal, but activities as identical as possible to those in human tissues. This implies efficient substitution of the yeast reductase by the human enzyme and adjustment of its level, as well as that of cytochrome b_5, to fit as closely as possible conditions found in human liver. Overexpression of the mammalian reductase in yeast appeared more difficult than overproduction of the yeast equivalent. A possible cause is the strong secondary structure present within the 5-end of the human reductase ORF. This difficulty can be compensated by the high copy number of plasmid-based expression systems as reported by Eugster et al. [12]. Nevertheless, in our laboratory, we prefer to pursue the more versatile strategy involving genomic integration similar to the one involved in the construction of the W(R) strain.

A difficulty, raised by the single copy expression requirement (a consequence of genome

integration), was to find another way to compensate for the low expression level of the human reductase. This was achieved specifically by removing by site-directed mutagenesis the secondary structure originally present on the human reductase cDNA without changing the amino acid sequence [13]. This procedure resulted in the W(hR) strain which exhibits a gene structure at the *YRED* locus (locus YHR042w on chromosome VIII encoding yeast microsomal reductase) very similar to that of W(R) except for the production of the human reductase. Based on the NADPH-cytochrome *c* oxidoreduction assay, the specific P450 reductase activity in W(hR) microsomes was found to be rather low (20-fold) in comparison to the level in W(R) [13]. However, upon transformation by an expression vector for human P450 3A4, a surprisingly high P450 specific activity was observed provided that a suitable buffer was used and that human cytochrome b_5 was added (Fig. 2). Interestingly, the ionic strength dependency of the 3A4 activity was found optimal for a physiologic buffer (Fig. 3). In such conditions, low amounts of human reductase support more efficiently P450 3A4 activities than high amounts of the yeast reductase. A detailed analysis of this observation is currently in progress to understand by which mechanisms reductases originating from different species can have such strong differential recognition for various human P450s.

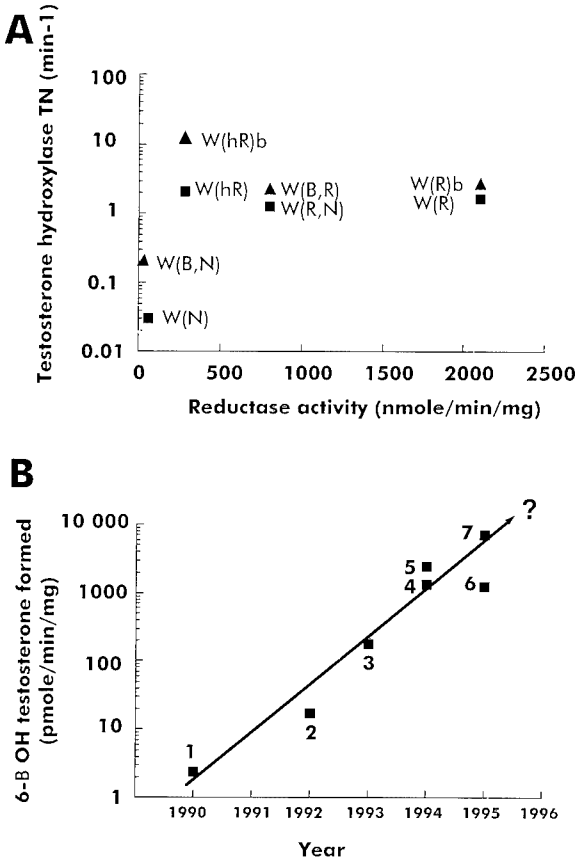


Fig. 2. (A): Human P450 3A4 and NADPH-P450 reductase activities in the various generations of engineered yeast strains (see references for detail). P450 activities are given as turnover numbers (nmol metabolite formed by nmol P450 per min) when reductase activity is expressed as nmol cytochrome *c* reduced per min and per mg microsomal protein. (B): Progress in the P450 3A4 metabolic activity expressed per mg yeast microsomal proteins. Years indicated correspond to the date of first observation of the corresponding activity value. These data, being expressed as specific activity (per mg protein), include both progresses in the increase of turnover numbers and in the P450 specific contents. (1) W(N), wild type; (2) W(B,N), express human b_5; (4,5) W(R) and W(R,N), overexpress yeast reductase; (3) W(B,R), overexpress yeast reductase and human b_5; (6,7) W(hR), express human reductase; (5,7) W(xx)b, purified b_5 added. All strains were transformed by pH3A4-V60.

3. Simulation of human multistep drug metabolism

The preceding results illustrate that the yeast system is particularly well suited to the qualitative and quantitative simulation of a single step P450-dependent metabolism. In fact, the strategy of genomic integration used previously can be easily extended to the coexpression of some phase II enzymes, such as epoxide hydrolase, making possible the simulation in a single yeast cell of a multistep metabolism involving the sequential combination of phase I and phase II reactions. This strategy was particularly illustrated in our laboratory in the case of the multistep metabolic activation of the environmental pollutant: benzo[*a*]pyrene [14]. When microsomes prepared from W(R) cells transformed by an expression vector for human P450 1A1

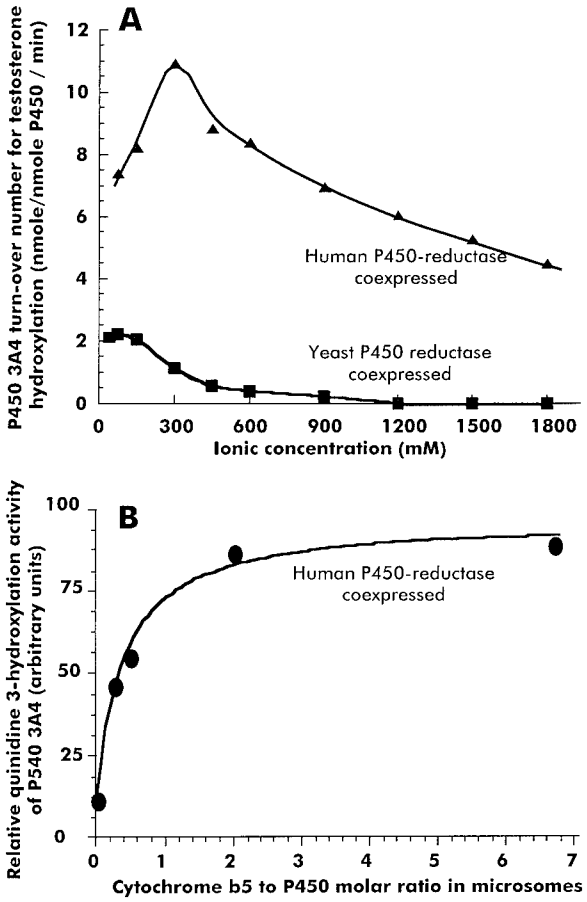


Fig. 3. (A): Dependence of the human 3A4 activity on the nature of the reductase coexpressed (human or yeast) and on the incubation buffer ionic strength. Strains were respectively (W(hR)b pH3A4-V60) and (w(R)b pH3A4-V60). The point at 300 mM corresponds to the following composition: phosphate, 50 mM; carbonate, 12 mM; chloride, 30 mM; potassium, 132 mM; sodium, 12 mM; pH 7.3. Incubation temperature is 37°C and turnover number is calculated based on the carbon monoxide difference spectra of P450 in the yeast microsomes. Specific contents in the used microsomes were: P450 3A4 (600 pmol/mg), human reductase (100 pmol/mg), yeast reductase (1000 pmol/mg). (B): Effect of human cytochrome b_5 saturation on P450 3A4 activity in yeast microsomes. Activity values are given as per cent of the saturation value.

were incubated with benzo[*a*]pyrene, formation of 3-hydroxy-, 9-hydroxy- and quinone derivatives was easily detected. Nevertheless, no formation of 7,8-, 4,5- or 9,10-dihydrodiols was observed due to the lack of endogenous epoxide hydrolase activity. The integration of the expression cassette coding for microsomal epoxide hydrolase was performed at the *YRED* locus, leading to W(E) strain, in a way fully similar to that utilised for integration of the human cytochrome b_5 coding sequence. After mating W(E) with W(R), the resulting W(E,R) diploid strain expressed human epoxide hydrolase and overexpressed yeast P450 reductase. After transformation by an expression plasmid for human P450 1A1, incubation of microsomes with benzo[*a*]pyrene led to the efficient formation of the expected diol product (resulting from a phase I-phase II coupling) and of the ultimate mutagen 7,8-dihydrodiol-9,10-oxide, as judged by the presence of its tetraol decomposition products and the formation of DNA adducts (Gautier et al., unpublished results). This demonstrates that the yeast expression system is not only a useful tool for the prediction of simple metabolism, but also is an efficient way to reproduce and analyse complex multistep metabolism leading to drug or pollutant toxic activation.

3.1. From yeast simulation to computer simulation of drug metabolism

The liver metabolism of a given drug may comprise a large number of different enzymes (particularly when several different P450 and phase II reactions are involved). Although the coexpression of a large number of heterologous enzymes in a single yeast is theoretically feasible using systematic genomic integration, such a solution would be generally of limited practical interest because of the large amount of work needed to integrate more than 3–4 heterologous activities together in a single cell. Only a limited number of standard activity combinations can be reasonably considered (for example one P450, cytochrome b_5, the reductase and one phase II activity). In addition, adjustment of the relative expression levels to fit the natural situation becomes rapidly technically demanding when a large number of components is considered. This last point is of importance when the aim is to take into account interindividual polymorphisms of expression of xenobiotic metabolism enzymes. To solve this problem we thus developed an

approach which associates yeast expression of a limited set of enzymes with numeric simulation to extrapolate metabolic predictions at different initial conditions. A first objective is to decompose complex enzymatic reactions, difficult to reconstruct to their full extent with the yeast system, into several sets of activities (coupled or individual) more simple to express. The second objective is to simulate the metabolic behaviour in human tissues, where relative isoenzyme concentrations and metabolite fluxes could be very different from those present in vitro in model systems.

The approach we have evaluated is illustrated by Fig. 4 in the case of benzo[*a*]pyrene metabolism and comprises the following steps: (i) when only stable metabolite intermediates are formed by independent enzymatic reactions, the major kinetic parameters (specific activity, half-saturation concentrations, half-inhibitory concentrations) are first determined using the yeast system to express separately the individual enzymes. The intermediate metabolites that cannot be easily obtained by chemical synthesis are prepared by large-scale bioconversion using the yeast system. Compounds thus prepared are purified by preparative HPLC and are used as substrate to quantify the subsequent enzymic steps; (ii) when unstable or reactive intermediates are involved (as illustrated in Fig. 4), coexpression of the enzyme forming the unstable intermediate and of a conjugating enzyme is performed. Only quantitation of stable metabolites is generally required. Kinetic parameters for unstable intermediates including non-enzymatic decomposition (for example conversion of oxides

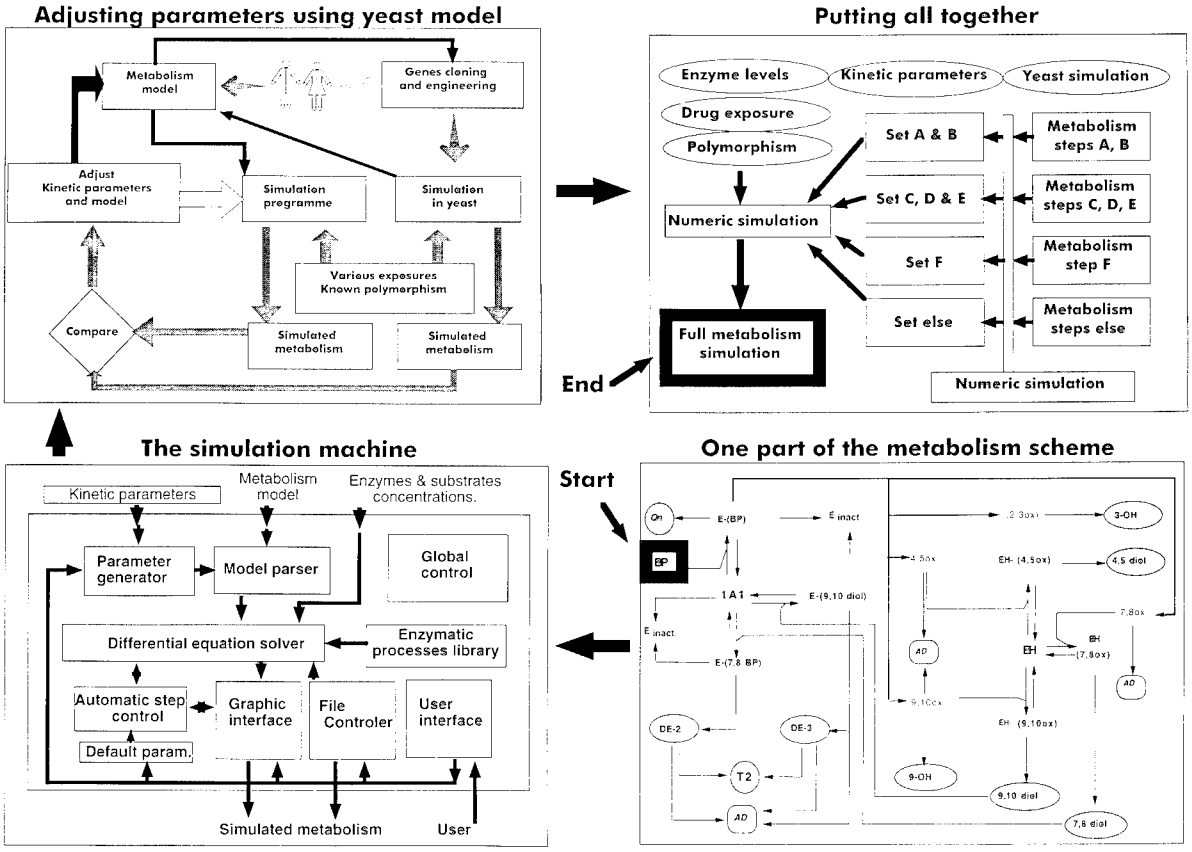


Fig. 4. Overall strategy for the association of yeast and computer simulations for metabolic prediction. The kinetic scheme illustrates part of the benzo[*a*]pyrene metabolism.

to phenols) or adduct formations are deduced from the simulation of the experimental formation of stable metabolites as a function of the concentrations of generating and conjugating enzymes (type (ii) experiments). We have experienced that models involving up to 30 different species (chemicals or enzyme complexes) are perfectly workable with such an approach. Finally, a complete metabolism model is built by putting together all enzymes (with their respective individual kinetic parameters) in the numerical model. Such simulations allow a calculation of metabolite patterns (including adduct formation) associated with any combination of enzyme concentrations and drug inputs. Consequences of interindividual polymorphisms can also be simulated. This approach was validated using the complete yeast system as a reference in the benzo[*a*]pyrene metabolism model, giving very consistent results between experimental and numerical simulations under a large variety of conditions. Results are also consistent with observed metabolic patterns in humans.

4. Conclusions

Yeast expression of xenobiotic acting activities is efficiently achieved using a strategy involving P450 expression from a plasmid-borne cassette and enzyme environment tailoring by multiple genomic integration. Depending on the aim (metabolite production or quantitative simulation of human behaviour) different redox environments may be optimal, leading to a series of engineered yeast strains adapted for each particular usage. Both high yield and high specific activities can be achieved making the yeast system extremely versatile. The system nevertheless has its limits when very complex metabolic pathways involving numerous combinations of phase I and phase II enzymes have to be reproduced. In such a case, the association of numerical simulation, with establishment of individual kinetic parameters from the yeast-expressed system, represents a real improvement allowing to take into account interindividual metabolic polymorphisms.

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Toxicology Letters

Genetically engineered mammalian cells and applications

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Abstract

In general, cells genetically engineered for stable and defined expression of xenobiotic-metabolizing enzymes are useful tools whenever a metabolism-related problem in toxicology and pharmacology is to be solved. It is the genetic and phenotypic nature of a given cell that determines its applicability. Mammalian cells have useful characteristics not given in bacterial, yeast or insect cells, which also may express xenobiotic-metabolizing enzymes. It is the problem to be solved and the question to be answered which determine the optimal choice for the best-suited expression system. There may even be subtle differences between mammalian cells of different species and organ origin, which might play a role in choosing a mammalian expression system. Thus, the level and specificity of the xenobiotic-metabolizing enzyme, the experimental testing conditions, and the biological endpoints present in a chosen cell are the most important criteria to be observed in the application of the mammalian expression systems.

Keywords: Biotransformation; Xenobiotics; Cytochrome P450; Heterologous expression

1. Introduction

In most cases, cytochromes P450 (CYP) are the key enzymes for the metabolism of xenobiotics, e.g. drugs and pollutants. Therefore, metabolism is of major concern for drug efficacy, drug safety, and the toxicological potential of pollutants and food contaminants. It is of fundamental interest to follow and to understand the metabolic fate of xenobiotics in detail. This implies the generation of metabolite profiles, the identification of the metabolically competent CYP isoform, and an assessment of the impact on biological endpoints related to metabolites. Animal studies and clinical trials have intrinsic limitations for studying these aspects in detail for several reasons. Detailed studies are hampered by the complexity caused by ever-changing factors and conditions in the individual organism and interindividual differences on CYP levels. Furthermore, there are species-specific differences in CYP in terms of substrate specificity and activity, tissue-specific expression, and regulation of gene expression. Those differences may not allow a direct extrapolation from animals to humans. Furthermore, access to animals and humans is restricted for political and ethical reasons. These are some of the reasons that make it worthwhile to apply technology for the development of in vitro systems for studying

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metabolism and metabolism-related problems. Cloning and expressing xenobiotic-metabolizing enzymes certainly is a rational and straight approach in the design of a metabolically competent and useful cell. These gene technological approaches for heterologous expression of xenobiotic-metabolizing enzymes are basically all the same. However, they differ in 2 important aspects: (i) the kind and construction of recombinant vectors and (ii) the chosen host cell for expression. Both aspects determine the range of application in toxicology and pharmacology.

Presently, heterologous expression of xenobiotic-metabolizing enzymes has been achieved in bacterial cells, e.g. *E. coli* and *Salmonella* strains, yeast, insect, and mammalian cells. Each of these expression systems has their advantages and limitations, depending on the experimental problem to be solved. Therefore, no such thing as the 'best' or 'ultimate' culture system will ever exist.

Although the expression levels achieved in mammalian cells are usually lower than in bacterial and yeast cells, there are several important reasons for engineering mammalian cells. These reasons are given by characteristic biological endpoints not present or different in yeast or bacterial cells, e.g. DNA repair, mutagenicity, chromosomal aberration, micronuclei formation, apoptosis, cytotoxicity, and many more. As mammalian cells differ in their phenotypes, they will perform differently for different biological endpoints. Therefore, it makes sense to have the same enzymes expressed in a variety of cell types of different tissue and species origin. The user has to make an educated choice for the one expression system that serves him and his problem best.

This laboratory has given preference to genetically engineering V79 Chinese hamster cells. V79 cells have a longstanding tradition in toxicological studies because they grow extremely fast with a doubling time of less than 12 h, and are very sturdy and reliable in their growth and genetic characteristics. However, metabolic capacity is usually low and restricted to a few conjugating enzymes. CYP have never been detected in these cells. Thus, genetically engineered V79 cells are defined for the cDNA-encoded CYP, and allow CYP-specific investigations without the need for a tedious enzyme purification in most cases.

2. Materials and methods

2.1. Construction of CYP-expressing V79 cell lines

The cDNAs for rat CYPs 1A1, 1A2, and 2B1 were cloned from cDNA libraries as full length cDNAs or as fragments and reconstructed for full length [1–3]. The cDNAs for human CYPs 1A1, 1A2, 2A6, and 2E1 were generously provided by Dr. F. Gonzalez, Bethesda, MA, CYP3A4 as expression vector LNC3A4 by Dr. J. Horbach, Utrecht, The Netherlands, orginally from Dr. Ph. Beaune, Paris, France. The very first applied SV40 Early promoter containing plasmid for CYP2B1 continues to serve as an expression vector by substitution of CYP2B1 cDNA for other cDNA, e.g. human CYP3A4 (Fig. 1A). For comparison, the same cDNA was also expressed in a vector containing the CMV promotor (Fig. 1B). V79 cells serve as host cells for all CYP recombinant expression vectors. These are attached growing cells. There are 2 variant V79 cell lines, which differ by expression of endogenous *N*-acetyltransferase. The variant cell line V79MZ does not express *N*-acetyltransferase and was obtained from Dr. C.F. Arlett, Sussex, UK and subcloned [4]. The variant cell line V79NH does express *N*-acetyltransferase and was received as subclone V79 379A from Flow Laboratories, Meckenheim, Germany. A nomenclature for the CYP-engineered V79 cell lines was chosen in agreement with the recommended CYP nomenclature [5,6]. The V79MZ cell line-based constructs are named for the species, e.g. h for human, and r for rat, and for the CYP isoform. Therefore, V79MZr2B1 denominates the cell line expressing rat CYP2B1, and V79MZh1A1 the cell line for human 1A1.

2.2. Characterization of CYP-expressing V79 cells

Cells were selected for stable integration and expression of CYP cDNAs, which is shown by

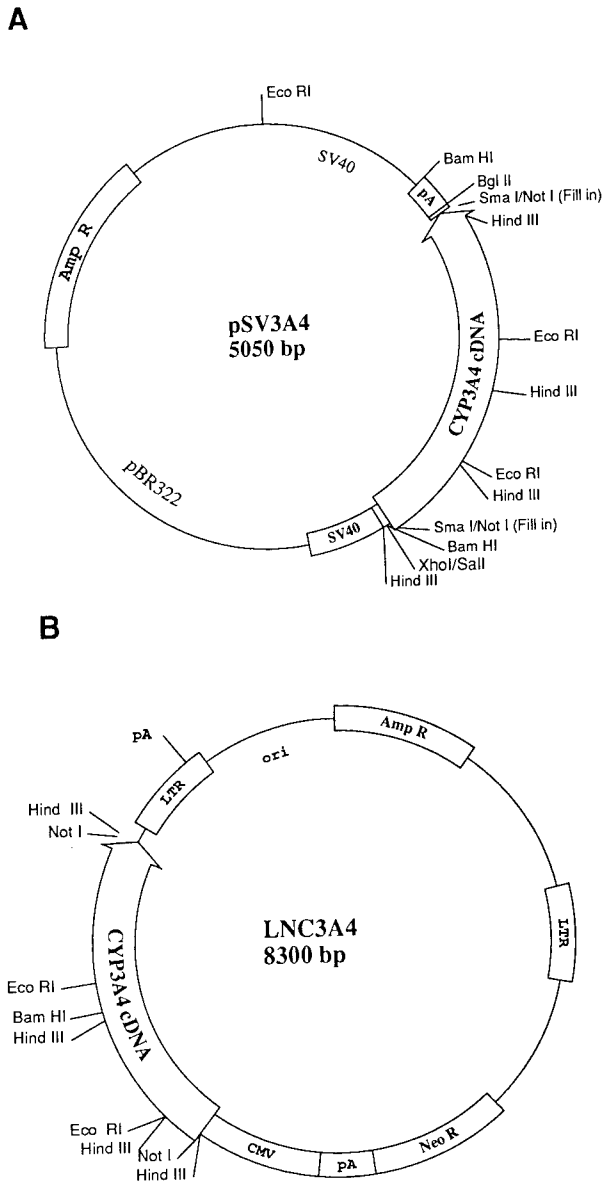


Fig. 1. Recombinant expression vectors for human CYP3A4 for stable expression in V79 cells, containing the SV40 Early promotor (A) or the CMV promotor (B).

Southern, Northern, and Western analysis. Enzyme activities are determined by standard procedures using benzo[*a*]pyrene, several resorufins, and coumarin as substrates. Characterization for each of the engineered cell lines has been described in detail [1–3,6,7] (Schneider et al., pers. commun.). CYP-expressing V79 cells are also identified by in situ immunofluorescence using CYP-specific antibodies [8].

3. Results

3.1. Integration and expression of CYP cDNA in V79 cells

Most engineered V79 cell lines contain 1 integrated copy of the CYP cDNA in their chromosomal DNA, as is the case for V79 cell line expressing human CYP3A4. This is shown by restricting the genomic DNA with *Bgl*II, which has no recognition site in the CYP3A4 construct. The inserted cDNA may be further characterized with additional restriction enzymes, which have recognition sites within the construct for further analysis of the integrity of the integrated cDNA expression cassette, as shown for restriction with *Hind*III.

Efficient expression of the CYP cDNA in V79 cells was shown by Western analysis, e.g. human CYP3A4 (Fig. 2). In all Western analyses performed so far, no endogenous CYP expression was ever detected in the parental V79 cells.

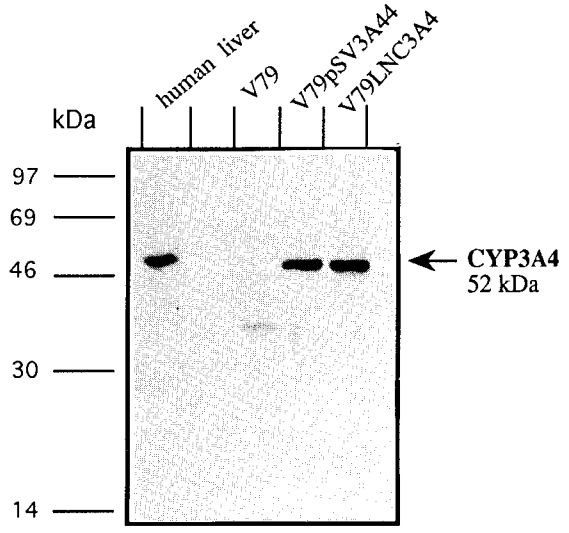


Fig. 2. Western analysis of cell lines V79MZh3A4 in comparison to authentic human CYP3A4 and V79 parental cell.

3.2. CYP content and activity levels in V79 cells

For comparison of results from different expression systems it is necessary to know the CYP content in terms of mol per mg total protein, which is measured by a CO difference spectrum. However, considerable amounts of CYP are needed in a sample for this kind of measurement. This is a major obstacle for mammalian expression systems with relatively low expression levels. It was only recently possible to perform this measurement for V79 cells by growing 10^{10} cells. The V79MZh1A1 cells were analyzed by the CO difference spectrum and were found to contain between 5 and 15 pmol human CYP1A1 per mg microsomal protein, depending on the processing procedure (Vermeulen et al., in preparation). From comparative Western analyses it is estimated that the CYP content is in the range of 10–50 pmol per mg total cellular protein [3]. The range of enyzme activities in V79 cells were found to be between 5 and well over 100 pmol/min/mg total protein, assessed with substrates thought to be more or less CYP isoform specific, such as ethoxyresorufin, benzo[*a*]pyrene, coumarin, and chlorzoxazone.

3.3. Application of CYP-expressing V79 cells

CYP-expressing V79 cells were applied in several toxicological and pharmacological studies, when CYP-dependent metabolic activation was important. This was the case for mutagenicity studies on several polycyclic aromatic hydrocarbons [9], and on cytotoxicity and micronuclei formation studies, e.g. on aflatoxin B_1 (Fig. 3). CYP-dependent metabolite profiles were generated, e.g. for caffeine [9] and for polycyclic aromatic hydrocarbons [10].

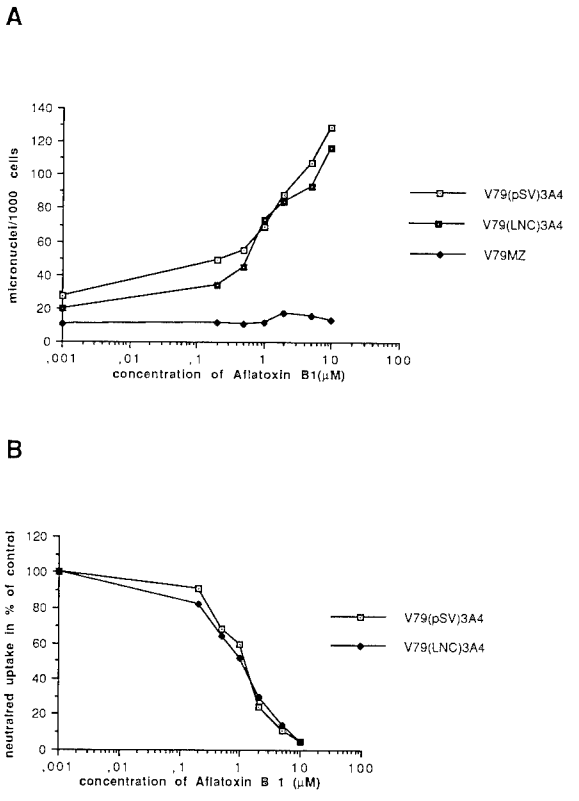


Fig. 3. Aflatoxin B_1-induced toxicity in V79MZh3A4 cells in comparison to parental V79MZ cells for micronulcei formation (A) and cytotoxicity (B).

4. Conclusion

By now, almost 9 years have passed since the beginning of our CYP expression project at the end of 1986. In the meantime, several CYP expression systems have been established, and a lot of experience has been gained on the performance of these systems. Our results on the genetically engineered V79 cells justify the initial approach by applying the SV40 expression vector and the V79 cells as the host cells for the CYP cDNAs. Expression of CYPs appears to be stable in V79 cells and sufficient for many metabolism-related studies. The cell biological characteristics of the engineered V79 ceils are basically the same as for the V79 parental cells, i.e. fast growth, suitable biological endpoints, and no endogenous CYP activity. Therefore, it is worthwhile to expand the V79 cell battery at least for the most important CYPs, referred to as the 'big eleven' responsible for metabolic activation of more than 99% of all xenobiotics.

At the same time, others have achieved the expression of CYPs in bacterial, yeast, insect and additional mammalian cells. Each of these systems stand on their own rights, as they all differ in fundamental biological features in general and in CYP expression levels in particular. It is the scientific question or problem to be solved that

should dictate the most appropiate choice among all of the expression systems.

No matter which of the heterologous expression systems is given preference to, they all have in common that CYPs are expressed, which would be difficult to obtain in large quantities from native tissues. Secondly, genetically engineered systems are defined for CYPs and may be experimentally applied under standardized conditions. Therefore, genetically engineered CYP expression systems should be considered to be valuable analytical tools in order to dissect the complexity of the in vivo situation. In this context there is a clear need for genetically engineered expression systems. It is difficult to envision that primary human hepatocytes will be as suitable models as the CYP expression systems, no matter how much the culturing conditions of the human hepatocyte systems are optimized.

Acknowledgements

We thank Hildegard Mäser for excellent technical assistance, and the Deutsche Forschungsgemeinschaft for funding.

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Salmonella strains and mammalian cells genetically engineered for expression of sulfotransferases

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Abstract

Rat and human sulfotransferases (STs) were expressed in *his*$^-$ *S. typhimurium* strains. These new bacterial strains detected various mutagens which are difficult to recognize in traditional test systems, including benzylic alcohols derived from polycyclic aromatic hydrocarbons, hycanthone and 1′-hydroxysafrole. STs were also stably expressed in V79 Chinese hamster cells, which do not express endogenous ST and are suitable for the detection of genotoxic effects. Positive responses in these test systems were observed with various benzylic alcohols, including benzo[*a*]pyrene-7,8,9,10-tetrols. We demonstrate that a few reactive sulfuric acid conjugates are efficiently detected as genotoxicants only when generated directly within the indicator cell.

Keywords: Benzylic alcohols; Heterologous expression; Hycanthone; Safrole; *Salmonella typhimurium*; Sulfotransferase; V79 cells

1. Introduction

Sulfation by sulfotransferases (STs) (EC 2.8.2) is a common terminal step in the metabolism of xenobiotics. The typical and desired products are hydrophilic, charged conjugates which are readily excreted in the urine or bile. However, sulfation of some substrates, such as benzylic and allylic alcohols or aromatic hydroxylamines and hydroxamic acids, leads to the generation of electrophilic products, which may covalently bind to tissue macromolecules and induce mutations and tumors [1]. Compared to cytochrome P450-mediated activation of chemical carcinogens, the sulfation pathway has received little attention. This is especially true for studies using in vitro systems to assess bioactivation. One of the possible reasons for this lack of investigation is that sulfate conjugates may not readily penetrate cell membranes. This property is required for their excretion to avoid reabsorption and results from their negative charge at physiological pH values. Therefore, reactive sulfate conjugates may not be reliably detected when added or generated outside the detector cell in test systems. However, in

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a few cases, spontaneous substitution reactions, e.g. with chloride anions, may lead to the formation of lipophilic reactive species which readily penetrate membranes [2,3].

Since STs are not naturally expressed in the indicator cells of common in vitro mutagenicity test systems [4], we constructed ST-proficient bacterial and mammalian indicator cells using gene transfer techniques. *Salmonella typhimurium* TA1538 and Chinese hamster V79 cells were used as recipient cells. In this paper we report on initial experiences in mutagenicity investigations using these new test organisms.

Rats, as well as humans, express multiple forms of cytosolic sulfotransferase [5,6]. On the basis of their preferred substrates and their amino acid sequences, they are classified into phenol (or aryl) STs (PSTs), estrogen STs (ESTs) and hydroxysteroid (or alcohol) STs (HSTs). In humans, only single forms of EST and HST are known, whereas 2 different PSTs can be distinguished, the phenol-sulfating or thermostabile form (P-PST) and the catecholamine-sulfating or thermolabile form (M-PST). In the rat, a larger number of different STs has been detected, including several forms of HST and PST. In the present study we have expressed 3 human STs (HST, P-PST, M-PST) and 2 rat STs. The rat STs used are the major HST (HSTa), a female-dominant enzyme, and PST-IV (also frequently abbreviated AST-IV), a male-dominant enzyme. We use the prefixes h and r for human and rat, respectively, to the abbreviations, when the origin of the ST is not obvious.

2. Materials and methods

2.1. Molecular cloning of STs and expression in Salmonella typhimurium

The cloning of the cDNAs of hHST, hP-PST, hM-PST, rHSTa and rPST-IV, and their expression in *Escherichia coli* XL-1 Blue cells using the pKK233-2 vector has been previously reported [7–9]. The plasmids were adapted to the restriction enzymes of *S. typhimurium* LT2 by passaging them through the restriction-deficient, but methylation-proficient *S. typhimurium* strain LB5000 [10], before transformation of the *his* strain TA1538. The new strains were given the designations of the parental strain, followed by the abbreviation of the expressed enzyme, e.g. TA1538-hHST.

2.2. Stable expression in Chinese hamster V79 cells

The construction and characterization of the rHSTa-proficient cell line V79-rSTa-1 has been described elsewhere [11].

2.3. Mutagenicity assay in bacteria

The transformed bacterial strains were grown overnight in the presence of ampicillin (100 μg/ml) to reinforce the maintenance of the recombinant plasmid, and isopropyl-β-D-thiogalactopyranoside (1 mg/ml) to activate the *lac* promoter of the recombinant ST gene. Mutagenicity was tested using a 20 min-preincubation assay with 5-fold higher than usual cell density [9]. Bacterial cytosol was prepared by ultrasonication, dialyzed against buffer and supplemented with the cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) [9].

2.4. Sister chromatid exchange (SCE) in V79-derived cell lines

The same protocol which we previously described for parental V79 cells [12] was used. Since the rHSTa-encoding vector was co-transfected into V79 cells together with a puromycin resistance marker, a control cell line was used, which only expressed this resistance marker (V79p).

3. Results

1-Hydroxymethylpyrene (for a review see Ref. [13]), a metabolite of an environmentally relevant carcinogenic polycyclic hydrocarbon, was inactive in the parental strain TA1538, but was mutagenic in all 5 strains which express ST (Fig. 1, left panel). Stronger effects were observed in the strains expressing HST than in those expressing PST. Rat HSTa was somewhat more effective than human HST (initial slopes of the dose-response curves: 750 and 260 revertants per nmol, respectively). Hycanthone, a schis-

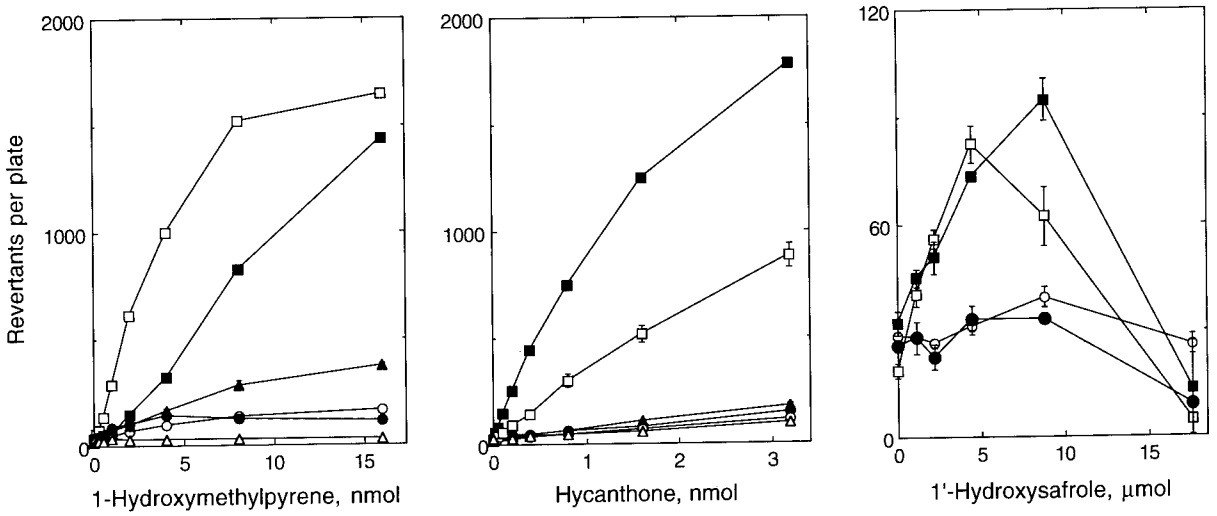


Fig. 1. Mutagenicity of 1-hydroxymethylpyrene, hycanthone and 1′-hydroxysafrole to *S. typhimurium* TA1538 (△), and TA1538-derived strains which express rat HSTa (□), human HST (■), rat PST-IV (○), human P-PST (●) and human M-PST (▲).

tosomacidal drug, was weakly mutagenic in the parental strain TA1538 (24 revertants per nmol). These effects were virtually unaffected when the PSTs were expressed, but were strongly enhanced when the HSTs were expressed in TA1538 cells (Fig. 1, middle panel). With hycanthone, human HST was more efficient (1400 revertants per nmol) than rat HSTa (250 revertants per nmol). 1′-Hydroxysafrole, a metabolite of safrole, a carcinogen which occurs naturally in various food flavors, was not mutagenic to TA1538 (data not shown) nor to the isogenic strains expressing hP-PST or rPST-IV; however, 1′-hydroxysafrole was mutagenic to both strains expressing rHSTa or hHST (Fig. 1, right panel). The effect of 1′-hydroxysafrole was much weaker and required much higher concentrations than those of 1-hydroxymethylpyrene and hycanthone. The number of induced revertants per nmol 1′-hydroxysafrole amounted to 0.015 and 0.009 in strains TA1538-rHSTa and TA1538-hHST, respectively.

In order to compare the efficiencies of endogenous and exogenous activations, the compounds were tested for mutagenicity (i) directly in the ST-proficient strain TA1538-hHST, and (ii) in the ST-deficient strain TA538 in the presence of PAPS- and chloride-supplemented cytosol from strain TA1538-hHST. 1-Hydroxymethylpyrene was strongly mutagenic under both experimental conditions. Although 6-hydroxymethylbenzo[*a*]pyrene was more potent than 1-hydroxymethylpyrene to strain TA1538-hHST (1500 versus 210 revertants per nmol), it showed only minimal mutagenicity in the presence of the exogenous activating system (Fig. 2). Only when very high concentrations of substrate and cytosol from TA1538-hHST were used, could the mutagenic effects be enhanced (data not shown). Like 6-hydroxymethylbenzo[*a*]pyrene, hycanthone showed low responsiveness towards exogenous activation by cofactor-supplemented cytosol from strain TA1538-hHST (data not shown), but was strongly mutagenic when directly tested in this strain (Fig. 1).

The V79-rSTa-1 cell line was used for the study of the genotoxicity of a series of compounds which are structurally related to 1-hydroxymethylpyrene. The benzylic carbocation generated from 1-hydroxymethylpyrene is stabilized by the same aromatic system as those from the bay-region dihydrodiol-epoxides of benzo[*a*]pyrene, the most thoroughly investigated carcinogenic polycyclic aromatic hydrocarbon. Moreover, the hydrolysis products of the benzo[*a*]pyrene dihydrodiol-epoxides, the 7,8,9,10-

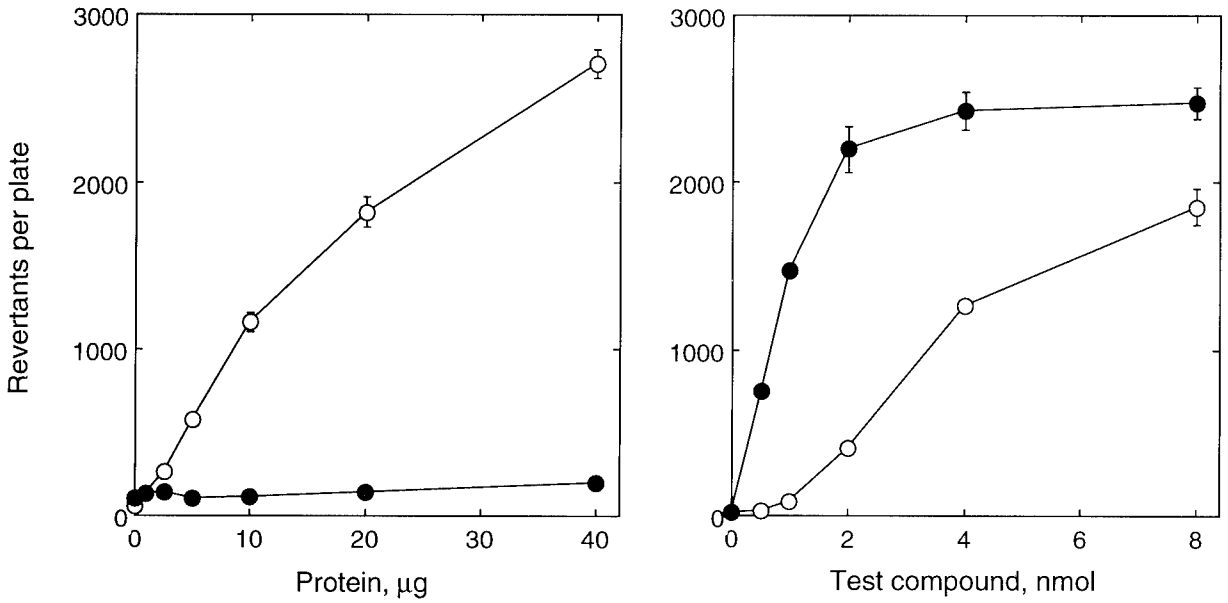


Fig. 2. Mutagenicity of 1-hydroxymethylpyrene (○) and 6-hydroxymethylbenzo[*a*]pyrene (●) using exogenous or endogenous activation by human HST expressed in *S. typhimurium*. Left panel: a fixed amount (43 nmol) of test compound was tested for mutagenicity to strain TA1538 in the presence of PAPS and varying amounts of cytosol from strain TA1538-hHST. Right panel: varying amounts of the benzylic alcohols were tested directly in strain TA1538-hHST in the absence of an exogenous metabolizing system. Values are means and S.E. of 3 plates. Where no error bar is shown, it falls within the symbol.

tetrahydrotetrols, that contain 1-hydroxymethylpyrene in their structure, might be reactivated by sulfation of the hydroxyl group in position 10 and then form the same DNA adducts as do the dihydrodiol-epoxides. For this reason, 1-hydroxymethylpyrene, benzo[*a*]pyrene-7,10/8,9-tetrol (trans-hydrolysis products of *anti*-benzo[*a*]pyrene-7,8-diol-9,10-oxide) and benzo[*a*]pyrene-7/8,9,10-tetrol (cis-hydrolysis products of *anti*-benzo[*a*]pyrene-7,8-diol-9,10-oxide) were tested for genotoxic activity in ST-proficient cells. 1-(1-Hydroxyethyl)pyrene and 10-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, which structurally are derived from 1-hydroxymethylpyrene by the extension and anellation of the side chain, were also included in this series. All 5 compounds increased the frequency of SCE in V79-rSTa-1 cells (Fig. 3), whereas they were inactive or much less active in V79p cells. At low substrate concentrations, 1-hydroxymethylpyrene showed the strongest effects, and the tetrols showed the weakest effects. However, the tetrols were also much less cytotoxic than the other compounds, and therefore could be tested at higher levels. With benzo[*a*]pyrene-7/8,9,10-tetrol, the relatively low activity per concentration unit could be compensated for by the concentration which could be tested, so that it elicited the highest absolute increase in the frequency of SCE among all congeners.

4. Discussion

Both in *Salmonella* and V79 cells, heterologous expression of STs led to strong genotoxic effects with compounds which were inactive or weakly active in the parental, ST-deficient cells. It is therefore concluded that these compounds were activated by metabolic sulfation and that the expressed STs were enzymatically active and supplied with cofactor in the recombinant cells.

Exogenously added PAPS- and chloride-fortified cytosol preparations from strain TA1538-hHST efficiently activated 1-hydroxymethylpyrene to a mutagen. In previous studies, other exogenous sources of ST activity have also been

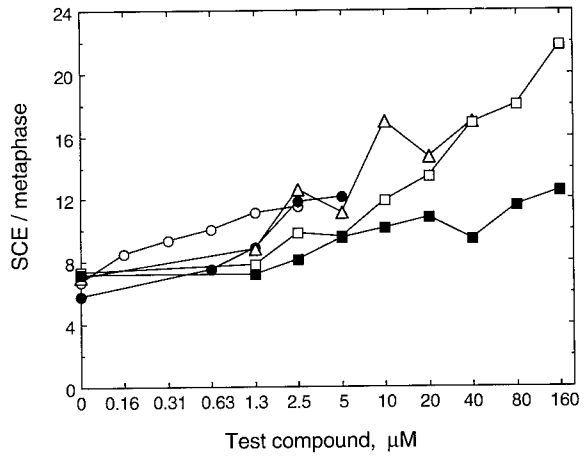


Fig. 3. Induction of SCE in V79-rSTa-1 cells (expressing rat HSTa) by 1-hydroxymethylpyrene (○), 1-(1-hydroxyethyl)pyrene (●), 10-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene (△), benzo[*a*]pyrene-7,10/8,9-tetrol (■) and benzo[*a*]-pyrene-7/8,9,10-tetrol (□). All compounds were tested up to the limits of toxicity. Values are means of 25 metaphases and normalized for 22 chromosomes. S.E. was usually <15% of the mean. Benzo[*a*]pyrene-7/8,9,10-tetrol induced SCE also in ST-deficient V79p control cells (expressing the puromycin selection marker only), but the activity was only about 1/10 of that observed in V79-rSTa-1 cells. All other compounds were inactive in V79p cells (increase in the number of SCE per metaphase of ≤1 at all concentration levels).

very efficient with this compound [11,14], whereby 1-chloromethylpyrene, which is spontaneously formed from 1-sulfoxymethylpyrene, appears to be the major active species [2,13]. The positive response with 1-hydroxymethylpyrene demonstrates that the exogenous hHST was active. However, the same enzyme preparations added to the cell medium only supported the mutagenicity of 6-hydroxymethylbenzo[*a*]pyrene and hycanthone with very low efficiencies, although these compounds were stronger mutagens than 1-hydroxymethylpyrene when hHST was expressed in the indicator bacteria. It is therefore concluded that only a very small proportion of the active metabolites of 6-hydroxymethylbenzo[*a*]pyrene and hycanthone reaches the intracellular target when they are generated outside the indicator cell. Although these findings were made using bacterial indicator cells, it is probable that they can be confirmed in mammalian cells, since it is the aim of phase-II metabolism to reduce the ability of the compounds to passively penetrate cell membranes.

We did not detect mutagenic effects of 10-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene or benzo[*a*]pyrene-tetrols in *Salmonella* using external activating systems ([14] and unpublished results) and have not yet tested them in the new bacterial strains. However, they showed ST-dependent genotoxicity in V79-derived mammalian cells. The tetrols contain 2 benzylic hydroxyl groups, in the 7 and 10 positions, but it is not known which of them are sulfated. The observation that 10-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene was substantially more effective in the induction of SCE than 7-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene may suggest that the 10 (bay-region) position is sulfated. It remains to be investigated to what extent reactivation of tetrols contributes to the high biological activity of dihydrodiol-epoxides. An analogous reactivation by sulfation has been reported for 3,4-dihydroxy-3,4-dihydrocyclopenta[*c,d*]pyrene, the hydrolysis product of cyclopenta[*c,d*]pyrene-3,4-oxide [15].

Some STs are expressed with high tissue and cell selectivity. The HSTs are primarily expressed in the liver. Selective expression of the activating enzyme together with limitations in the penetration into other cells may explain the organotropism of the investigated compounds. 6-Hydroxymethylbenzo[*a*]pyrene is a potent hepatocarcinogen [1]. Also safrole exerts its carcinogenic effects in the liver [1]. The medical use of hycanthone is severely limited by its hepatotoxicity [1]. Other STs are expressed at high levels in extrahepatic tissues, such as gut and brain. For example, the specific activity of sulfation of terbutaline in human duodenum is about 30 times higher than in the liver, and high levels were also detected in the other sections of the small and large intestine [16]. It appeared that hM-PST was involved in this activity.

The new bacterial and mammalian indicator cells will allow the testing of chemicals for ST-dependent bioactivation as well as the identification of the involved ST forms.

Recently, additional forms of STs have been

stably expressed in V79 cells, a second form of rat HST (rHST20) (I. Bartsch, A. Czich and H.R. Glatt, unpublished), rat hydroxamic acid ST (rST1C1) (I. Bartsch, Y. Yamazoe and H.R. Glatt, unpublished), hM-PST and hP-PST [17], and hHST [18].

Acknowledgements

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The hazards of synthetic (anthropogenic) chemicals

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Abstract

Currently, there is a highly politicized debate on the comparative risks of naturally occurring and synthetic chemicals. Since humans are often exposed to complex mixtures of both classes of chemicals, the real-world value of this debate is unclear. However, in any such comparison, it is important to realize that some naturally occurring substances – such as lead or tobacco – have been so altered or disturbed by human activity that they are best considered as products of human activity, or anthropogenic. Reasons for concern over synthetic and anthropogenic chemicals include: persistence and propensity for long-term storage in biota and the environment; structural similarity to endogenous biomolecules; and additivity to natural hazards.

Keywords: Risk assessment; Synthetic chemicals; Carcinogens; Xenoestrogens; Metals; Dioxins; PCBs

1. Introduction

To debate the relative hazards of synthetic as compared to natural compounds is at one level somewhat trivial. In the real world, humans and ecosystems encounter exposures to both classes of toxicants, often in mixtures. However, there are serious elements to this topic, most particularly because of the renewed efforts of some to elevate this question to the highest priority in discussions of environmental health [1]. A current National Research Council committee is charged with considering the public health significance of naturally occurring carcinogens, an issue raised by Ames and others, who frequently criticize the allocation of testing and regulatory resources towards synthetic chemicals. That naturally occurring compounds and conditions can be extremely toxic (usually acutely) is beyond dispute. What is more relevant to consider is whether current research and regulatory policy neglect these natural risks by placing undue emphasis and 'unfair' restrictions upon synthetic risks. The policy implications of this issue are at least twofold: first, there is proposed legislation that mandates comparative risk assessment as a necessary component of health and safety policies, explicitly requiring that regulators must consider the risks of chemicals, such as pesticides, in the context of such natural hazards as lightning, floods, asteroid impacts, or exposure to natural mutagens such as aflatoxin (S 343, 104th Congress; see [2,3] for comments). There has been one ingenious attempt to undertake a comparison of the carcinogenic risks of aflatoxin and the pesticide/growth regulator Alar, which found that under conditions of anticipated dietary exposures, Alar may be riskier [4]. Second,

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some have demanded that equal investments in testing programs be given to mutagens and other toxins in food in the National Toxicology Program cancer bioassay [5]. There have been compelling discussions of phytoestrogens as potentially important xenoestrogens (or environmental endocrine disruptors) [6]. Are these demands scientifically reasonable or merely politically disingenuous, and what is their likely impact upon environmental research and public health policy?

In discussing this topic, at the outset I propose an expansion of the definition of synthetic chemicals. Ordinarily, we understand this category to denote those compounds produced by human activity, that are otherwise not found in nature (or possibly found in trace amounts, such as some dioxins). I propose to add to this category compounds that may be of natural origin but whose presence or physical-chemical form in the biosphere has been substantially perturbed by human activity such that human exposures have been greatly magnified. For instance, tobacco is a natural plant, but the extraction of tobacco and other elements and processing into smokable or chewable material has presented humans with an anthropogenic hazard not encountered from tobacco in its natural state. Also in this category the useful but toxic elements lead, cadmium, and mercury are properly classified. Over the past 5000 years, as shown in Fig. 1, anthropogenic exploitation of lead has multiplied concentrations of lead in long-term environmental sinks such as undisturbed soils, sediments, ice, and corals [7,8]. This enrichment of the human environment has resulted in logarithmic increases in what are now considered 'background' levels of lead in blood, even in remote populations. As shown in Fig. 2, our blood lead levels are between 10–100 times that estimated to have been the levels of lead in blood in preindustrial humans [9]. Blood lead levels in modern Americans are between 100 and 1000 times that of preindustrial populations (between 2 and 50 μg/dl, as compared to 0.01–0.02 μg/dl).

These enrichments are comparable to more recent changes in concentrations of dioxins in human tissues and sedimentary records [10,11]. This period coincides with the development of

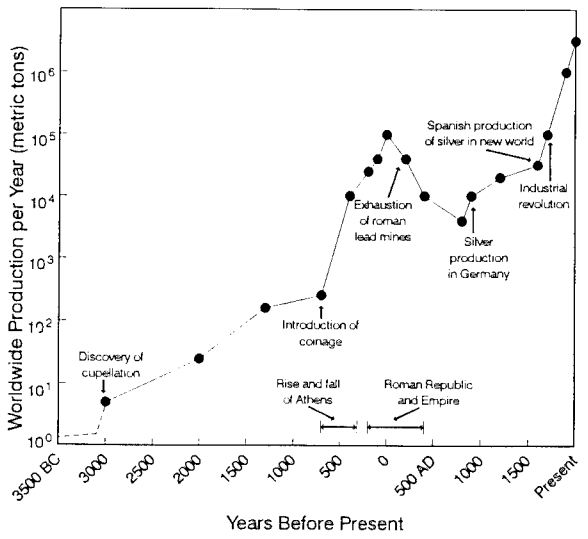


Fig. 1. History of anthropogenic lead exploitation from 3500 BC to the present (note that y-axis is logarithmic). Data from [7].

the synthetic organic chemical industry, and the discovery and production of many interesting chlorinated derivatives of benzene and phenol [12]. Thus although humans have not 'created' lead, in some type of reverse alchemy, we have substantially changed its natural patterns of location and disposition with the result that both likelihood and intensity of exposures are increased. For that reason, it is more useful to expand the category of synthetic compounds,

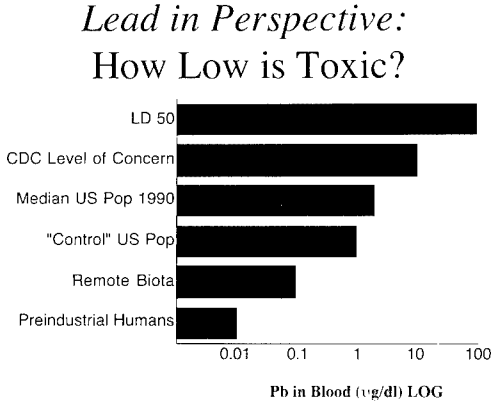


Fig. 2. Estimated and measured blood lead levels in human populations, from pre-industrial times to the present Data from [8,9].

such as dioxins and PCBs, to include anthropogenically perturbed substances like lead, mercury, and cadmium.

2. Reasons for increased concern for synthetic/anthropogenic compounds

There are several general reasons to suspect that the category of synthetic and anthropogenic chemicals may contain greater risks, or a greater number of risky substances, than the category of natural chemicals. Synthetic chemicals may possess some of the following characteristics that can increase their risks:

- persistence and long-term metabolic storage;
- structural similarity to endogenous compounds;
- 'stickiness' to receptors, channels, or other biomolecules;
- additivity or synergism with natural toxins.

2.1. Persistence and metabolic storage

Some synthetic compounds are resistant to both metabolic degradation and physical/chemical breakdown. This is generally not the case with natural compounds, such as plant estrogens, natural mutagens, or neurotoxins of plant or animal origin. Many of these are subject to enzymatic processing, rapid hydrolysis or photolytic breakdown. This is not surprising, given their origin in biota. In addition to persistence, some synthetic compounds are also partitioned into lipid compartments or stored in mineralized tissue, unlike most natural toxins that are hydrophilic in nature (because they are manufactured and secreted by cells). It has also been suggested by some [13] that humans have evolved in the context of these natural toxic compounds, particularly those found in diet, and that we have acquired through selection efficient biological mechanisms, such as inducible enzymes, to detoxify many of them.

These two characteristics have resulted in the ubiquitous presence of displaced elements, like lead, and some highly persistent lipophiles throughout the biosphere. The stable isomers of the organochlorines – PCBs, PCDDs, PCDFs, dieldrin, lindane, chlordane, heptachlor, and the DDT metabolite DDE – are among these compounds. Recent surveys have demonstrated the ubiquitous presence of these compounds in the diets and tissues of even remote populations. For example, Inuit populations in Arctic Quebec have breastmilk concentrations of PCBs, PCDDs, and PCDFs that are much higher than those found in breastmilk sampled from urban women living in Quebec city [14]. The nature of biomagnification through food webs, including humans as top predators, increases the likelihood of exposures for carnivorous or omnivorous consumers, such as humans and marine mammals. Lipophilicity also results in partitioning and secretion of some of these chemicals in breastmilk, a high fat source [15]. Several recent surveys have suggested that a relatively high proportion of human exposures to dioxin-like compounds may come via breastfeeding [16].

Several toxic metals that have been subject to extensive anthropogenic transfers from their natural crustal locations are stored in mineral compartments within organisms. In humans, most of the body burden of lead is found in the skeleton; strontium, aluminum, cadmium, and mercury are also stored in this organ [17]. There is evidence that these metals may be toxic to bone; however, of greater potential concern is the possible mobilization of this integrated store during such physiological conditions as pregnancy, lactation, renal disease, calcium deficiency, menopause, and aging [18].

It should be noted that even in the case of nonpersistent chemicals, if uses and releases are sufficiently dispersive and intense, increased concentrations of these chemicals can be found almost as ubiquitously as the persistent compounds and elements discussed above. As shown in Table 1, global measurements taken of methylene chloride, a nonpersistent volatile organic solvent in widespread use, demonstrate that this chemical can be measured in air samples worldwide, although there are clear 'hotspots' associated with expected sources such as waste disposal and industrial facilities [19].

Table 1
Levels of methylene chloride in air at selected locations

Location	Concentration ($\mu g/m^3$)	Notes
	Mean	
Wiltshire, UK	0.1 (range)	Dec/Jan 74/5; 13 samples
Northern Italy	<5–100	Samples from outside 14 houses and one office block
Netherlands	1.4–14 (range)	Three sites: background, suburban, near sources; 1979–81
Arctic: clear air	0.08	Spring 1984; 30 samples from aircraft
haze	0.11	
Norwegian Arctic	0.26	July 1982
Spitzbergen	0.29	March 1983
California	7.2	Mean of means, max. mean 20 $\mu g/m^3$
US: rural and remote	45	Summary data 1970—80:
remote urban	630	718 samples
suburban source dominated	270	127 samples
US landfills	3200	Survey of levels in gases from landfills
Japan: 1979	0.25–5.3 (range)	25 of 46 positive samples
1980	0.30–2.8 (range)	47 of 135 positive samples
1981	0.01–19.8 (range)	99 of 101 positive samples
Northern hemisphere	0.13	December 1981 sample date
Southern hemisphere	0.07	December 1981 sample date

Data from Ref. [19].

2.2. Similarity to endogenous molecules

Sometimes deliberately and sometimes inadvertently, humans have synthesized and used molecules with structural similarities to potent endogenous chemicals. Pesticides, fungicides, and plant growth regulators are examples of compounds deliberately designed to resemble endogenous bioactive molecules, such as neurotransmitters and hormones. Biotechnology may yield further variants upon this theme with even greater homology to natural regulators of cell/cell communication, growth and senescence. Although at one time it was hoped that we could enjoy selective protection from these designer toxins either because of assumed qualitative differences between humans and pests such as insects, fungi, and nematodes or because of dose, this security has often proved to be illusory. Moreover, the unrecognized potential for some of these agents to accumulate in the biosphere (e.g. the organochlorine pesticides that act upon neurotransmitter-regulated ionophores [28]) has destroyed any short-term immediate advantage of dose restriction at the time of application.

The most notorious example of an inadvertent 'mimic' of an endogenous molecule is probably TCDD. As is now clear from the elegant knockout studies of transgenic mice [29], the endogenous acceptor/transducer molecule for dioxin plays an important role in development, and possibly other cell functions. Unfortunately for us and other species, the synthetic molecule TCDD appears to have the best 'fit' to this receptor and to induce downstream events of altered gene transcription with very great potency [20]. Some other chlorinated hydrocarbons may also bear inadvertent resemblance to identified endogenous hormones, such as estrogen, thyroid hormone, retinol, and androgens [21]. It may be that the anthropogenically-disturbed toxin cadmium exerts its toxicity by 'mimicking' zinc at critical molecular sites, such as binding to metallothionein.

2.3. Stickiness

In some cases, these toxicants may be more potent than the endogenous compounds they replace or augment. This increased potency can result from simple toxicokinetics, since in some cases these synthetic mimics are more slowly

metabolized or cleared than the endogenous compound, which is usually cleared or catabolized rapidly in order to permit rapid changes in cell state. As pointed out by Birnbaum and colleagues in studies of the dioxins, slower toxicokinetic clearance can confer biological activity even upon compounds that show very little activity in acute in vitro studies [20].

In addition, some of these compounds may be 'stickier' at acceptor sites because of slight differences in physical chemical characteristics. Lead, for instance, appears to bind at many calcium-binding sites at least in the nervous system. However, because of its much greater mass/charge ratio, lead may have greater affinity (or slower off rates) than calcium or zinc at these sites, as appears to be the case for calmodulin and protein kinase C [22]. This is why lead can displace zinc, atom for atom, in the enzyme aminolevulinic acid dehydrase, but zinc cannot displace lead until much higher concentrations are added (Silbergeld, unpublished data). Increased 'stickiness' may increase the ability of these xenobiotics to compete with natural ligands or compounds at binding sites, and may cause weak agonists to exert antagonist effects.

2.4. Additivity

Finally, there is a simple consideration of additivity. No matter how large or significant the load of risks from natural compounds, they are added to by the increasing input of synthetic compounds and anthropogenically-disturbed toxins. That is, a human exposed to phytoestrogens in plants may now be also exposed to chlordecone, DDT, PCBs, and other xenoestrogens of synthetic origin, as well as the increased loadings of plant estrogens halogenated during the anthropogenic activity of pulp bleaching. Some of these combined exposures may be inconsequential; some may add no net risk, as suggested by Safe [6]. However, at least some are likely to be additive, and a few may be synergistic. While this argument does not detract from the suggestion that the natural toxins should also be tested and evaluated, it provides some rationale for exerting a stringent 'gatekeeper' function over the deliberate addition of new chemicals into the 'sea of mutagens' (to quote Ames) and other toxins in which we already exist.

3. Should we be concerned?

Some have suggested that since lifespans are increasing and in many countries other health indicators indicate improvement in terms of the incidence and prevalence of certain diseases, that the relatively recent increases in synthetic chemicals cannot be damaging human health. (It should be noted in passing that surveys of trends in some wildlife populations, such as the Great Lakes, do not support such optimism [23].) A considerable debate exists about the evidence from data such as cancer mortality and morbidity trends over the past 60 years [1,13]. While such general data cannot confirm or exclude relationships between exposures and risk, it should be remembered that it was trend data of this type that first alerted public health officials and physicians to the very great risks of smoking, when lung cancer death rates in men began to escalate in the early 1930s, some two decades after smoking became a prevalent social behavior.

Temporal trends in incidence of asthma and other respiratory conditions have been correlated with changes in air quality, related to such pollutants as NO_x, sulfur oxides, ozone and particulates. In some cases, correlative evidence exists from prospective studies of persons exposed to fluctuations in concentrations of these pollutants [24].

More recently, evidence has been offered to suggest possible increases in trends for outcomes related to reproduction and development. As shown in Table 2, the Center for Disease Control (CDC) Birth Defects Monitoring Program has provided some evidence for increases in certain structural defects likely to be largely if not entirely independent of changes in diagnosis or surveillance [25]. Of even greater public concern, has been evidence presented for declining sperm counts and quality over time [6]. The studies indicate that sperm counts have decreased, particularly in younger men, over the past decade

Table 2
Trends in the incidence of selected birth defects, from international birth defects monitoring system, 1983–1991

Defect combination with anotia/microtia	No. of cases/year		
	1983–1985	1986–1988	1989–1991
Hydrocephalus plus cleft palate	0	1	6
Microcephalus plus cleft palate	0	0	4
Minor facial defects plus cleft palate	0	3	14
Cleft palate plus anal atresia	1	2	5
Cleft palate plus deformations	0	5	9
Minor facial defects plus cardiac defects	7	6	13
Minor facial defects plus deformations	3	7	14

Data from Ref. [25].

particularly, but possibly with a steady rate for a much longer period. The correlates and antecedents of these events are unknown, but different patterns among countries may further elucidate potential hypotheses for epidemiological and toxicological study.

4. Conclusions

Both natural and synthetic/anthropogenic compounds can present risks to human health, dependent upon dose and variations in susceptibility among exposed individuals and populations. However, to demand equal treatment of both categories is not sensible scientifically or good public policy. Scientifically, there is reason to be relatively more concerned about many synthetic compounds, particularly those that are persistent, metabolically stored within biota, designed to resemble biomolecules, and more active than their natural analogues at defined biological sites within cells and organs. The first four characteristics – persistence, metabolic storage, deliberate design, and increased bioactivity – are distinctive of some synthetic, as opposed to natural compounds. In terms of public policy, the advocates of 'equal treatment' for natural toxicants would flood our limited resources for toxicity testing, epidemiological surveillance, and risk assessment with thousands of natural compounds. This will not result in improvements in either policy or public health. Just as the NTP is highly selective in terms of its chemical nomination process [26], we must continue to exercise reasonable judgement in the evaluation of chemical and other risks in our environment. Insofar as human activity may continue to perturb naturally occurring substances, through exploitation of new elements (such as gallium, yttrium, and ytterbium, to cite three metals of great interest in microelectronics and superconducting, for which inadequate information on toxicity exists [27]) or the amplification or translocation of biological molecules through biotechnology from one species to another, then these compounds are rightly added to the subcategory of anthropogenic compounds. These deserve equal attention as those chemicals devised by human ingenuity. Given the continuing gross insufficiency of information on existing and new chemicals, it is imperative to continue to use the best judgment possible in selecting chemicals for extensive assessment, with the best scientific methods of toxicology and epidemiology, and in investing national resources in their sound management.

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Mycotoxins, general view, chemistry and structure

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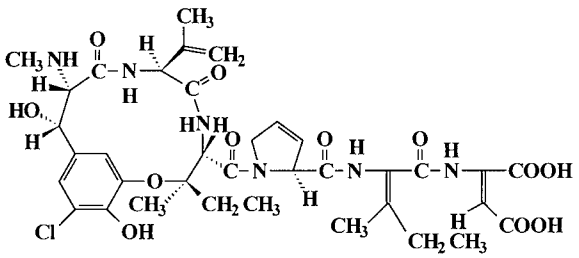
Abstract

Mycotoxins induce diverse and powerful biological effects in test systems; some are carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic, and neurotoxic. Mycotoxins have been unambiguously linked to the etiology of several diseases in animals. The discovery of aflatoxins in the early 1960s led to the resurgence of interest in human mycotoxicoses; mycotoxins are now recognized as causal factors of primary liver cancer, ergotism and alimentary toxic aleukia. The fumonisins and ochratoxins are suspected of playing a role in the etiology of esophageal cancer and Balkan endemic nephrotoxicity, respectively.

Keywords: Mycotoxin; Aflatoxin; Mycotoxicosis; Hepatocarcinogen

1. Introduction

Mycotoxins are a structurally diverse group of mostly small molecular weight compounds, produced by the secondary metabolism of fungi, are ubiquitous in a broad range of commodities and feeds, and are toxic to mammals, poultry, and fish. In structural complexity, mycotoxins vary from simple C_4-compounds, e.g. moniliformin, to complex substances such as the phomopsins [1] and the tremorgenic mycotoxins [2].



Phomopsin A

Mycotoxins induce powerful and dissimilar biological effects. Some are carcinogenic (aflatoxins, ochratoxins and fumonisins), mutagenic (aflatoxins and sterigmatocystin), teratogenic (ochratoxins), estrogenic (zearalenone), hemorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ochratoxins), hepatotoxic (aflatoxins and phomopsins), dermotoxic (trichothecenes) and neurotoxic (ergotoxins, penitrems, lolitrems and paxilline), whereas others display antitumor, cytotoxic, and antimicrobial properties. The human ingestion of mycotoxins is due to the consumption of the mycotoxins in plant-based foods and their residues and metabolites in animal-derived foods, e.g. aflatoxin M_1 (AFM_1) in milk. Some of the toxic effects of mycotoxins are consistent with the characteristic symptoms seen in a number of human and animal diseases, such diseases caused by the ingestion of mycotoxins are called mycotoxicoses.

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The impact of mycotoxins on health depends on the amount of the mycotoxin consumed, the toxicity of the compound, e.g. acute or chronic (e.g. carcinogenic) effects, the body weight of the individual, the presence of other mycotoxins (synergistic effects) and other dietary effects [3]. Hsieh [4] maintained that all of the following criteria have to be satisfied to link a mycotoxin to a specific human disease: occurrence of the mycotoxin in food supplies; human exposure to the mycotoxin; correlation between exposure and incidence; reproducibility of the characteristic symptoms in experimental animals; similar mode of action in human and animal models.

The global nature of the mycotoxin problem is based on well-documented human mycotoxicoses such as ergotism in Europe, alimentary toxic aleukia (ATA) in Russia, acute aflatoxicoses in South and East Asia, and human primary liver cancer (PLC) in Africa and South East Asia. Ochratoxin A (OTA) is suspected to play a role in Balkan endemic nephropathy (BEN) in Yugoslavia and chronic interstitial nephritis (CIN) in North Africa.

In nature most cereal grains, oil seeds, tree nuts, and dehydrated fruits are susceptible to fungus contamination and mycotoxin formation. Under laboratory conditions at least 300 mycotoxins have been produced by pure cultures of fungi and chemically characterised. Fortunately, only about 20 mycotoxins are known to occur in foodstuffs at significant levels and frequency to be of food safety concern. These toxins are mainly produced by 5 genera of fungi: *Aspergillus, Penicillium, Fusarium, Alternaria, and Claviceps.*

The mycotoxins produced by these fungi are: *Aspergillus* toxins, aflatoxin B_1, G_1, M_1, OTA, sterigmatocystin and cyclopiazonic acid; *Penicillium* toxins, patulin, OTA, citrinin, penitrem A (PA) and cyclopiazonic acid; *Fusarium* toxins, deoxynivalenol, nivalenol, zearalenone, T-2 toxin, diacetoxyscirpenol, fumonisins and moniliformin; *Alternaria* toxins, tenuazonic acid, alternariol and alternariol methyl ether; *Claviceps* toxins, ergot alkaloids.

Domestic animals are more frequently exposed to mycotoxins. Some mycotoxicoses, e.g. lupinosis and diplodiosis are linked to the ingestion of feeds contaminated by *Phomopsis leptostromiformis* and *Diplodia maydis (Stennocarpella maydis)*, respectively.

2. Ergotoxins

Ergotism is probably the oldest known mycotoxicosis. Human outbreaks associated with *Claviceps purpurea* contamination of rye flour were widespread in central and northern Europe in the Middle Ages, e.g. the epidemic of the year 944 in Aquitaine and Limoges in France which killed 40 000 people. As a human disease, ergotism has almost been eliminated. However, there are still some isolated cases, for example King reported an outbreak of ergotism in Wollo, Ethiopia, affecting close to 150 people [5]. In Wollo the causal factors seem to have been the preceding prolonged wet season, and the large amounts of wild oats (*Avena abyssinica*) growing with the barley.

Two characteristic forms of ergotism can be distinguished in man, namely gangrenous ergotism and convulsive ergotism. Many of the victims of gangrenous ergotism regarded the burning sensation in their limbs as a divine punishment, and it was described as the Holy Fire, St. Anthony's Fire and *feu sacré.*

Claviceps sclerotia contain a diverse array of chemicals with about 100 compounds identified [6]. The lysergic acid derivatives are subdivided into simple acid amides (e.g. ergomitrine) and peptides which are further subdivided into ergotamine, ergotixine and ergoxine groups.

3. Aflatoxins

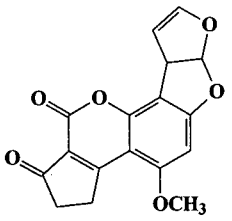
The epoch-making discovery during 1960 of the aflatoxins, a group of closely related hepatocarcinogenic bisdihydrofurano metabolites, produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*, led to the resurgence of interest in all aspects of mycotoxicology. Aflatoxin B_1 (AFB_1) is the most carcinogenic of the aflatoxins; it is the most commonly occurring

aflatoxin and has been said to be the most potent hepatocarcinogen to rats and mice. AFM_1 excreted in the milk of lactating cows has toxic properties similar to AFB_1; it is therefore of great public concern, particularly to young children.

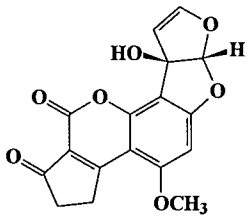
The potent hepatocarcinogenicity of the aflatoxins led to extensive studies of their carcinogenic properties, detailed information was obtained on their world-wide occurrence in foods and feeds, and their putative role as causal factors for human PLC. The liver is the primary target organ in different animal species, however, tumors of other organs have also been observed in aflatoxin-treated species [7], as well as a wide variation in sensitivity between and within species. The close response relationships between aflatoxin exposure and human liver cancer rates firmly established the role of aflatoxin in the etiology of human PLC in Africa and Asia [8]. IARC declared the aflatoxins in 1987 as human carcinogens, the classification was confirmed by re-evaluation in 1992.

The need to control aflatoxin exposure is based on 2 major concerns: the adverse short- and long-term effects of aflatoxin-contaminated commodities on human and animal health; the presence of aflatoxin residues or metabolites in animal tissues and milk used as human food.

The biological properties of the aflatoxins stimulated intensive research directed at the elucidation of the molecular and cellular mechanism by which the carcinogenic effects are induced. AFB_1 is transformed to its DNA binding form by the formation of the cytochrome P_{450}-induced epoxide and the subsequent reaction with the N^7 position of guanine; the AFB_1-DNA adducts are useful as biomarkers.



Aflatoxin B_1



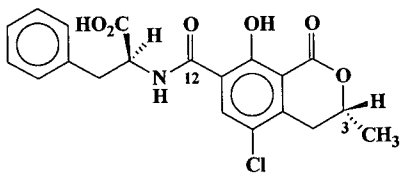
Aflatoxin M_1

Mutations in the nuclear protein p53, a tumor suppressor gene, were used in exploring the molecular etiology of aflatoxin's role in hepatocellular carcinomas (HCC). Sterling results were obtained in studies involving patients in China [9] and South Africa [10]; mutations were observed in codon 249 of the p53 gene of the patients. Virtually all the mutations resulted from G-C to T-A transversions and were localised at a single site in the codon (AGG to AGT). The uniformity of the nature of the observed base changes and their similarity to the mutations induced by AFB_1 in experimental systems support the interpretation that the tumors in patients may result from exposure to aflatoxins. However, Hsieh et al. found no correlation between the mutation at codon 249 of the p53 tumor suppressor gene and the amount of aflatoxin DNA adducts in liver tissue [11]. Wogan [12] maintained that the available evidence strongly suggested that human PLC is of multifactorial etiology with probable synergistic interactions between exposure to viral agents (hepatitis B and C) and chemical agents such as AFB_1 in populations exposed to both. Within a population group differences of susceptibility exist to aflatoxin carcinogenesis; these differences may be genetically linked.

4. Ochratoxins

The ochratoxins comprise a polyketide-derived dihydroisocoumarin moiety linked via its 12-carboxyl group by an amide bond to L-β-phenylalanine. OTA and its ethyl ester are the most toxic compounds. Ochratoxin B (OB), the dechloro derivative of OTA, is essentially nontoxic.

Aspergillus species are associated with OTA



Ochratoxin A

production in tropical areas, whereas OTA-producing *Penicillium* species thrive and can produce OTA in a colder climate with temperatures as low as 5°C. OTA became regarded as a very important mycotoxin since it plays a major role in the nephropathy occurring in swine (Danish porcine nephropathy) and poultry.

The occurrence of OTA in several plant and animal products has been extensively reported. OTA contamination is typically associated with grain stored in the temperate climate of Europe and North America. The kidneys are the organs most susceptible to OTA; it can cause both acute and chronic kidney lesions. OTA principally acts on the first part of the proximal tubules in the kidney and induces a defect in the anion transport mechanism on the brush border of the proximal convoluted tubular cells and basolateral membranes [13]. In addition, the immunotoxic, carcinogenic and teratogenic effects of OTA are well established.

The vast difference in the toxicity and hydrolysis rates of OTA and OB remained a significant challenge. We therefore studied the conformations of OTA and OB in the solid and solution state. Both compounds crystallise in the space group $P2_1$ [14]. Only minor differences were observed for the torsional angles of the side-chain and no significant differences in bond lengths and angles were observed for these 2 toxins. IR and NMR spectroscopic studies of OTA and OTB also point to the same conformation; the conundrum related to the difference in toxicity remains unresolved.

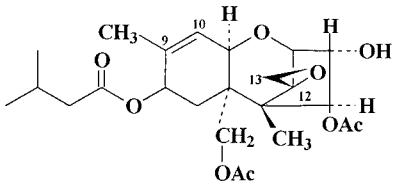
There is increasing epidemiological evidence that OTA is related to BEN and urinary tract tumors (UTT) in humans, predominantly observed in females showing an extreme geographic and familial aggregation [15]. Much attention is currently directed to the significantly higher frequencies of CIN in a North African population (Tunisia) and the possible role of high levels of OTA in the disease. The similarity between the clinical symptoms of some Tunisian patients with CIN and those with BEN warranted a closer scrutiny for the possible role of OTA. In Tunisia the elevated blood OTA levels were linked to CIN [16] (P.H. Bach, pers. commun.), in which case 95% of the people suffering from nephropathy are OTA positive with blood concentrations higher than 90 ng/ml in several cases.

5. Trichothecenes

The trichothecenes comprise a group of closely related mycotoxins called *sesquiterpenoids*. All the naturally occurring toxins contain an olefinic bond at C-9,10 and an epoxy group at C-12,13.; the latter characterises them as 12,13-epoxy-trichothecenes. Dangerous levels of trichothecene mycotoxins can occur in mouldy grains, cereals and agricultural products [17]. The tricho thecenes are produced by various species of *Fusarium, Trichoderma, Cephalosporium, Verticimonisporium* and *Stachybotrys*.

The genus *Fusarium* contains important mycotoxin-producing species [18]. Toxinogenic *fusaria* have been implicated in human health diseases such as ATA [19], Kashin-Beck disease, akakabibyo (scabby grain intoxication) and esophageal cancer, as well as in a number of animal diseases such as skin toxicity, bone marrow damage, haemorrhagic and estrogenic syndrome (zearalenone), and equine leukoencephalomalacia (ELEM, fumonisins).

Over the period 1942–1947 more than 10% of the population in the Orenburg district, close to Siberia, were fatally affected by consuming overwintered millet, wheat and barley. Wannemacher et al. [20] and Yagen et al. [19] reported that a disease whose symptoms are similar to those of ATA were in fact reported in Russia from the beginning of the 19th century. ATA has become associated with the consumption of food made from grain which remained unharvested under snow and became mouldy from contamination



T-2 Toxin

with a variety of micro-organisms. Symptoms of ATA include vomiting, diarrhoea, skin inflammation, leukopenia, multiple haemorrhage, necrotic angina, sepsis and exhaustion of bone marrow. Yagen et al. concluded that the trichothecene mycotoxin, T-2 toxin, was responsible for an ATA-like syndrome in cats.

Sapa-Associated Press (February 19, 1993) reported that poisoned wheat had killed 24 people and was threatening thousands more in war-torn southern Tajikistan. It was possible that the 'tainted wheat' referred to mould-spoiled wheat, and that humans were dying from trichothecene-related mycotoxicoses.

6. Tremorgenic mycotoxins

An inspection of the structures of various mycotoxins reveals nitrogen to be a fairly common constituent. In case of viridicatumtoxin and the fumonisins the nitrogen moiety is most likely derived from an ammonia source. The role of amino acids in the biosynthesis of the cytochalasins, chaetoglobosins, rhizonin A and phomopsin A is evident [21].

Tryptophan (Trp) is a common constituent of many secondary metabolites, several affecting the central nervous systems, such as the ergot alkaloids. Trp is the biogenetic precursor of the cyclopiazonic acids [22], tremorgenic substances such as fumitremorgens A and B [23] and verruculogen [24]. In the structurally related metabolites, the brevianamides and austamides [25], Trp and proline contribute the dioxopiperazine part of the molecules. Trp is again a building block of the tetrapeptide metabolites, the tryptoquivalines, which contain in addition anthranillic acid, valine and methylalanine. L-Trp and L-histidine are the precursors of the dioxopiperazines, oxaline [26] and roquefortine [27], metabolites of *Penicillium oxalicum* and *Penicillium roqueforti*, respectively. Roquefortine, a compound which affects the central nervous system, is also produced by *Penicillium camemberti* and is as such a frequent contaminant of some cheeses.

We approached the structural elucidation of PA in a concerted manner by the simultaneous application of physical techniques (very high-field NMR spectroscopy, UV spectroscopy, CD and mass spectrometry) and biosynthetic studies, and employed the principles of biosynthetic architecture throughout the structural studies [2].

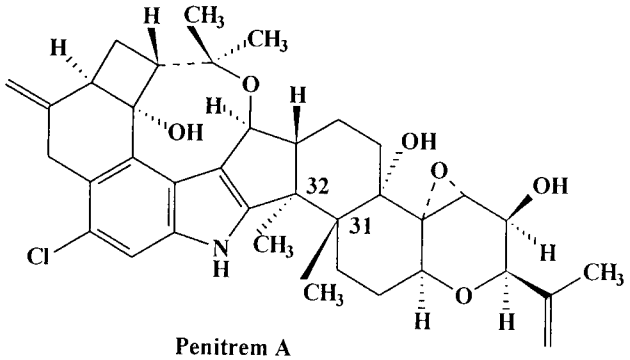
A brief survey of the structural properties of the fungal tremorgens, namely penitrems, janthitrems, lolitrems, aflatrem, paxilline, paspaline, paspalicine, paspalinine and paspalitrems A and B, reveals their close biogenetic relationship. In the case of aflatrem and paspalitrems A and B, a C_5 unit is attached to the paspaline-type structure [2].

7. Fumonisins

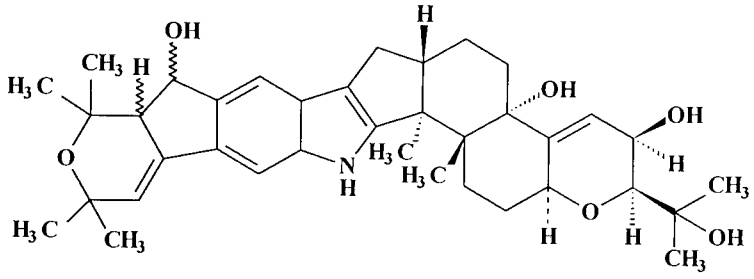
The ubiquitous fungus, *Fusarium moniliforme* Sheldon, a common contaminant of corn throughout the world, has been implicated in various animal and human diseases [28], owing to its toxicity and carcinogenicity [29].

Contamination of corn with *F. moniliforme* has been associated with human esophageal cancer in the Transkei part of Southern Africa [30], and in China [31], as well as with field outbreaks of ELEM in many countries such as Egypt, South Africa and the United States of America [28] and pulmonary oedema in swine [32]. ELEM is a fatal neurological disease of horses, characterised by liquefactive necrosis of the white matter of the brain. ELEM has been experimentally induced in horses by either supplementing their diets with *F. moniliforme*-contaminated corn or by the oral administration of fumonisin B_1 (FB_1), a toxin produced by *F. moniliforme* [33].

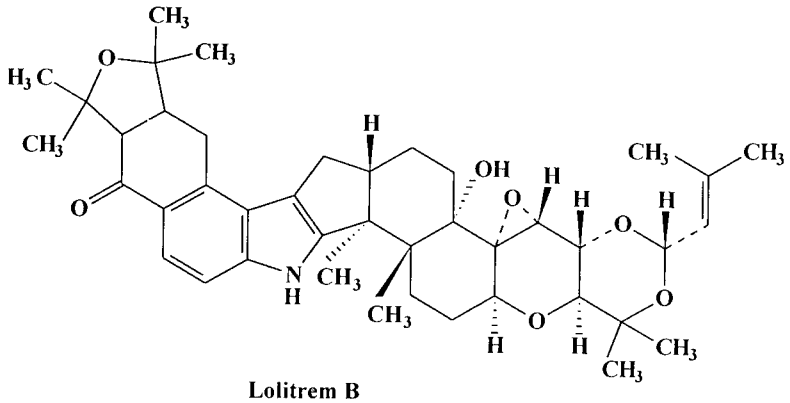
The health importance of *F. moniliforme* necessitated concerted efforts to isolate and characterise the toxins involved, as well as the development of analytical methodology to assess the level of contamination of natural foods and feeds by the toxin(s). Earlier studies led to the isolation of moniliformin and the fusarins [34]. Cawood et al. [35] developed an excellent method for the quantitative isolation of the fumonisins FB_1, FB_2 and FB_3. The availability of



Penitrem A



Janthitrem E

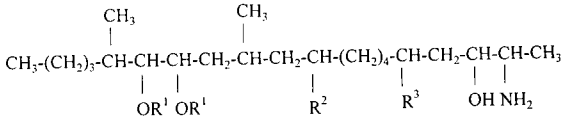


Lolitrem B

the fumonisins enabled researchers to study their unique biological properties. Alberts et al. [36] developed technology for the production of [^{14}C]FB$_1$. Fumonisin levels in food are statistically associated with an increased risk of human esophageal cancer in the Transkei [37].

The structural analysis of the fumonisins involved application of liquid secondary ion mass spectrometry of tetramethylfumonisin A$_1$ to give a protonated molecular ion at m/z 820 $(M+H)^+$. The interpretation of data from ^{13}C NMR spectra of tetramethylfumonisin A$_1$ led to a suggested empirical formula of $C_{40}H_{69}NO_{16}$ for tetramethylfumonisin A$_1$; acetylation of this sub-

stance gave a triacetate $C_{46}H_{75}NO_{19}$ (M^+945). Basic hydrolysis of this compound gave a neutral nitrogen-containing substance and propane-1,2,3-tricarboxylic acid. FB_1 and FB_2 were isolated by using a cancer promotion bio-assay [29], and were identified by detailed application and analysis of MS, ^1H and ^{13}C NMR data. The C-10 hydroxy group of FB_1 is replaced by a hydrogen atom in FB_2.



Fumonisin B_1: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$, $R^2 = OH$, $R^3 = OH$

Fumonisin B_2: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$, $R^2 = H$, $R^3 = OH$

Fumonisin B_3: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$, $R^2 = OH$, $R^3 = H$

Chemical structures of fumonisins B_1, B_2 and B_3

Analytical methods have been developed to monitor FB_1 contamination in various matrices, particularly corn and animal feeds [38–40]. Shephard et al. [41] developed an HPLC method for the analysis of the FB_1 and FB_2. It was the subject of a recently completed IUPAC collaborative study in which the reproducibility characteristics of the method were assessed [42]. Shephard et al. [43] developed technology for the liquid chromatographic determination of the mycotoxin FB_2 in physiological samples. The method was applied to the determination of the fate of FB_1 dosed to rats by gavage; of the dose given to the animals, over 90% was recovered unmetabolised in the faeces within 48 h.

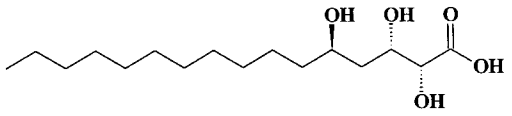
Shephard et al. [44] studied the distribution and excretion of a single dose of the mycotoxin [^{14}C]FB_1 in a non-human primate. The toxicokinetic data generated by the i.v. injection of FB_1 showed that the elimination of FB_1 from the plasma of monkeys can be characterised by an initial distribution phase followed by an elimination phase with a mean half-life of 40 min. The rapid elimination of FB_1 and the absence in the plasma of a major metabolite implies that direct measurement of FB_1 or its metabolites in blood will not be suitable to assess the extent of fumonisin exposure in animals and humans. Gelderblom et al. [45] used a short-term rat liver cancer initiating/promoter model to monitor the cancer-initiating activity of FB_1, FB_2 and FB_3 as well as the *N*-acetyl derivatives of FB_1 and FB_2 and their respective hydrolysis products, the polyols. They concluded that the intact molecule and the presence of a free amino group determined the carcinogenic activity of the fumonisins.

The fumonisins occur in many countries in a wide variety of corn-based matrices at, on occasions, relatively high concentrations. These data coupled with the toxicological evidence suggest that the fumonisins pose a potential threat to both animals and humans [39].

8. Future challenges

The identification of the toxins elaborated by *Stennocarpella maydis*, the study of their biological effects, and their natural occurrence remain one of the great challenges in mycotoxicology. Infestation of corn (maize) grain and cobs by this fungus is highly prevalent in Africa. Corn infected with this fungus is toxigenic to cattle, sheep, goats and poultry: in sheep and cattle diplodiosis is characterised by ataxia, paresis, salivation and constipation [46].

The potential danger to humans of corn contaminated with *S. maydis* was accentuated by the findings of Fincham et al. [47] that addition of *S. maydis*-infected corn (pure culture) to the food of omnivorous primates led to demyelination of nerves, atrophy, degeneration and necrosis of muscle and hepatitis. Steyn et al. [48] reported the discovery of diplodiatoxin, however, this compound is essentially non-toxic. Ackerman et al. [49] reported from cultures of this toxinogenic fungus, 2R,3S,5R-trihydroxyhexadecanoic acid, the fatty acid and its δ-lactone apparently contribute to the toxicity of the fungal culture.



2R,3S,5R-trihydroxyhexadecanoic acid

The fumonisins are an important group of mycotoxins owing to their world-wide occurrence in corn (maize) and corn products and their potent (carcinogenic) properties. Corn is an important human staple food, particularly in Africa – detailed studies on the toxicological effects of the fumonisins remain a top priority.

The biosynthesis of the aflatoxins was studied in great detail. However, very little progress was made in controlling the production of aflatoxin under natural agricultural conditions. The greatest challenge to plant breeders should be the development of cultivars resistant to infection by toxinogenic fungi.

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Mycotoxins: risk assessment and legislation

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Abstract

Mycotoxins are naturally occurring toxic secondary metabolites of fungi that may be present in food products. Several mycotoxins have been associated with and implicated in human and animal disease. Mycotoxins encompass a wide spectrum of different chemicals and they may affect many target organs and systems, notably the liver, kidney, the nervous system, the endocrine system, and the immune system. Much of our concern is about chronic effects at low levels of exposure, and several mycotoxins have been classified by the International Agency for Research in Cancer as human carcinogens or potential human carcinogens. Although these toxicants can never be completely removed from the food supply, it is possible through risk assessment to define levels that are unlikely to be of health concern. Ideally the risk assessment involves a complete toxicological assessment, an epidemiological assessment and an exposure assessment. However, in the risk management of mycotoxins it may be deemed necessary to take action before all this information is available. In this presentation recent risk management of the fumonisins will be compared with the situation for aflatoxins.

Keywords: Mycotoxins; Risk assessment; Fumonisins; Aflatoxins; Carcinogenicity

1. Introduction

Mycotoxins are fungal metabolites whose presence in feed and food crops cannot be completely avoided. Their presence can sometimes be reduced by making improvements in farming practices, such as by modifying seeds, providing better storage conditions, or by manufacturing processes. Surveys of food grains and food products conducted in Canada have shown the presence of low levels of several mycotoxins, such as aflatoxins, ochratoxin A, zearalenone, trichothecenes, patulin, ergot alkaloids, and recently the fumonisins. To find out whether small amounts of mycotoxins might pose a health risk to humans, one has to perform risk assessments, which look at the significance of human exposure to mycotoxins in terms of their toxicity or hazard.

Mycotoxins can be considered 'natural' contaminants. The principles applied to the hazard and risk assessment of mycotoxins are similar to those used for other chemical substances in the food supply, and were recently reviewed [1]. There is no evidence that humans have adapted to the presence of these natural chemical substances in food any more than to the presence of man-made chemicals, such as pesticides or other food chemical contaminants. Many of the mycotoxins have, in fact, been the

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cause of or have been associated with human and animal diseases.

For the risk assessment, the results of the exposure assessment (estimated probable daily intake or PDI) are compared with the hazard assessment (estimated tolerable daily intake (TDI) or equivalent) to indicate the degree of concern. There are many uncertainties in both the exposure assessment and the hazard assessment, which can therefore greatly influence the overall risk assessment [1,2]. Of necessity, regulatory risk assessments tend to err on the side of safety, until scientific data gaps have been resolved, and our understanding of the issues has improved. Thus the actual health risks, as a result of lifetime exposure to mycotoxins, are likely to be somewhat less than estimated. The risk assessment, therefore, represents a status document that needs to be updated as new data and new methods of extrapolating these data become available.

In Canada, when the estimated PDI of a mycotoxin exceeds its TDI, steps may be taken, under the authority of the Canadian Food and Drugs Act, to reduce the intake of that mycotoxin. These may range from completely removing the offending food from the market place to the establishing of tolerances (listed under schedule B-01-046 of the Food and Drugs Act and Regulations [3], and legally enforcable) or interim guideline levels (not listed in the Food and Drugs Act and Regulations, indicating Health Canada's interpretation of article 4a of the Act). Such actions limit the amount or level of the mycotoxin that can be tolerated in the raw food crop or in a finished food. Analytical methods have to be available to ensure compliance with a tolerance or a guideline level. Government regulations or guidelines for mycotoxins exist only with respect to aflatoxins and deoxynivalenol. In general, no tolerances or guideline levels have been adopted if the levels of mycotoxins in the food are consistently low, as measured through monitoring of food products, and have not been identified as a reason for health concern.

Similarly, to limit the indirect intake of mycotoxins, tolerances may be set for the maximum levels of mycotoxins that can be allowed in feed, to ensure negligible residues of mycotoxins and their metabolites in animal-derived food products [4]. These tolerances may be restricted to certain types of livestock (e.g. dairy versus beef cattle; layer versus broiler chickens). In the United States, where aflatoxin-contaminated feed has been a problem, it was found difficult to ensure adherence to the restrictions for different livestock species.

The presence of mycotoxins in food and feed commodities is related to climatic and other growth-related factors that influence the production of these secondary metabolites by the fungi. Thus there are considerable differences between various regions of the world as well as year-to-year fluctuations within countries. There are also considerable differences between countries and even within countries with regard to the intake of food commodities, thus making exposure assessments and therefore risk assessments country specific. Furthermore, because of considerations of other factors such as economic costs associated with lowering levels of mycotoxins, international trade aspects and what risks are considered tolerable, there are wide differences between countries in the risk management, including legislation, of mycotoxins. The various tolerances and guideline levels in effect for mycotoxins have been discussed [5].

In North America, the emphasis in recent years has been on less regulation or deregulation, and it is in this climate that any proposals for the risk management of natural toxicants such as mycotoxins need to be viewed. At the same time, there has been an increasing reliance on formal risk assessments to justify any proposed actions, or to identify important data gaps before taking action [6,7]. Nevertheless, risk management is necessary and possible when the perceived health concerns are considerable, even if the database is not complete. In this paper the recent interim risk assessment and risk management of fumonisins [8] will be compared with the earlier risk management of aflatoxins, which at present is undergoing review internationally.

2. Aflatoxins

Concern about the potential hazards posed by dietary aflatoxins started in the 1960s after some 100 000 turkey poults in Great Britain died as a result of aflatoxin exposure from their feed (for a review see [9]). When it became evident that aflatoxin exposure caused cancer in many species, most countries, including Canada, established various regulations for aflatoxin levels (either total aflatoxins or for aflatoxin B_1) in food and/or feed in order to limit exposure to this group of mycotoxins [5]. These initial regulations on aflatoxins were not based on the derivation of a TDI, but rather on a desire to keep levels as low as technologically feasible (basis for regulations in some countries), or 'free' of aflatoxins by not allowing residues above the analytical detection limit (basis for regulations in some other countries). The early prudent actions regarding aflatoxins by governments have been justified, since aflatoxin B_1 (AFB_1) has been found to be a potent genotoxic agent and carcinogen in many test systems and animal species. In the rat, development of hepatocellular carcinoma is preceded by the formation of liver foci and hyperplastic nodules [10]. Two of its metabolites, aflatoxin M_1 and aflatoxicol are also carcinogenic. Furthermore, epidemiological studies have shown an association between dietary aflatoxin exposure and the high incidence of primary liver cancer (PLC) in a number of African and South East Asian countries. The earlier studies were population-based (correlational, ecological) studies and only considered exposure to AFB_1. More recent studies have included an examination of the role of other factors, such as hepatitis B virus (HBV) infection [11]. Overall, recent studies indicate that HCC has a multifactorial etiology, in which HBV, AFB_1, alcohol consumption, smoking, and other environmental risk factors can all play a role [12]. Although there are additional risk factors, such as HBV infection, which may effect development of PLC, the strength and consistency of the epidemiological data indicate that AFB_1 is a probable human carcinogen. This view is consistent with the opinion expressed by the International Agency for Research on Cancer which concluded in 1987 that there is 'sufficient' evidence for the carcinogenicity of aflatoxins to humans [13]. This classification indicates that in the opinion of the IARC Working Group a positive relationship has been observed between exposure to aflatoxins and human liver cancer, in which chance, bias and confounding factors could be ruled out with reasonable confidence. Even when causality has been adequately determined, uncertainties remain regarding the quantitative aspects of the dose response curve, especially at low doses, and in the presence or absence of other risk factors. Analytical epidemiological studies (case-control and cohort) to better define the no-observed-adverse-effect level (NOAEL) in humans are ongoing. In these studies, exposure to both AFB_1 (based on intake 'at the plate' and an analysis of serum and urinary biomarkers) and HBV (based on HBsAg-carrier status) and disease outcome are determined at the level of the individual [14–17].

Because it is a genotoxic carcinogen, most agencies, including the Joint Expert Committee on Food Additives (JECFA) and the US Food and Drug Administration, have not set a TDI for AFB_1. Assuming there is a threshold for *non-genotoxic* carcinogens, and based on a safety factor approach, many agencies only estimate TDIs for those substances. In the risk management of *genotoxic* carcinogens, no threshold is presumed and it is recommended that levels of such substances should be as low as technologically feasible. However, it is important to give some guidance to the industry as to what levels they should aim for in setting technologically feasible limits. Because there is at present much debate about the varying mechanisms of action of non-genotoxic carcinogens and whether there are ultimately real differences in the health significance between these genotoxic and non-genotoxic carcinogens, we have used the term TDI in a broader sense. Thus we do not use this term only as the end point of a NOAEL/safety factor approach for non-genotoxic carcinogens or for other non-carcinogenic endpoints, but we

have, on occasion, also used it for genotoxic carcinogens. For this latter group we may use mathematical modelling, and determine the dose that is considered to pose a neglible risk to health, usually at a risk level of 10^{-5}, or other approaches of extrapolation such as using larger safety factors. Since many of the models are linear at low doses, the 2 approaches converge (at a risk level of 10^{-5}) when applying a safety factor of 5000 to a NOAEL or a statistically derived NOEL, such as a NEL or a Benchmark Dose [2]. Thus, based on the epidemiological data obtained in certain Asian and African countries, where the consumers may in addition be exposed to HBV infection, the estimated TDI for AFB_1, at a cancer risk level of 10^{-5}, ranges from 0.11 to 0.19 ng/kg body wt./day [17]. In the absence of endemic HBV infection, this range would probably be about 1 order of magnitude higher, but cannot be estimated more precisely from the presently available epidemiological data. Some other (possible) risk factors for PLC are alcohol intake, smoking, nutritional status, fumonisins in the diet and microcystins in the drinking water. It has not yet been possible to include the contribution of these other positive or possible negative risk factors.

Overall, the exposure to aflatoxins in Canada is one of the lowest in the world, and was estimated to be between 1 and 2 ng/kg body wt./day for 1–11-year old children eating peanuts or peanut butter [1,2]. Other age groups of Canadians have an even lower intake of AFB_1. The maximal intake estimate is about 10-fold higher than the estimated TDI, but since HBV infection is not endemic in Canada, the estimated intake of aflatoxins is unlikely to pose a health hazard. In Canada, a tolerance of 15 ng/g (total aflatoxins) for nuts and nut products is in effect [3], but this is at present under review. The tolerance for aflatoxins was set with respect to analytical methods available in 1976, and to levels which were technically achievable at that time. Since compliance to this tolerance is enforced by the Health Protection Branch (HPB), there is little likelihood that the Canadian population is subjected to peak exposure levels that significantly exceed the tolerance in those products. In fact, HPB monitoring data (1985–1987) indicate that on average the level of AFB_1 in commercial peanut butter is 1.62 ng/g. Under the auspices of Codex alimentaris, discussions are ongoing internationally as to whether Codex will recommend that the tolerances for nuts and nut products should be reduced now that technologically lower limits may be feasible and analytical detection limits have been reduced.

3. Fumonisins

The fumonisins, a group of mycotoxins, produced by *Fusarium moniliforme* and certain other *Fusarium* species, have recently been isolated and chemically characterized. Our program on the risk assessment of the fumonisins is part of an international effort to understand more about these toxins and to assess whether the levels of fumonisins found in foods pose a health risk, and whether guideline levels need to be established; the following summary is based on a recent evaluation [8].

The presence of *F. moniliforme* in feed has been related to at least 2 diseases in livestock that have a high fatality rate. The first disease is equine leukoencephalomalacia (ELEM), a disease which affects the liver and brain of horses, and which is known to have occurred in South Africa, the USA, Australia, Brazil, and elsewhere. The second disease is porcine pulmonary edema (PPE), a disease which affects the lungs, liver and kidneys of swine and which is known to have occurred in the USA. It has been possible to produce both of these diseases experimentally with pure fumonisin B_1 (FB_1). *F. moniliforme* also appears to play a role in the development of esophageal cancer in some parts of the world.

Considerable information is available on the mechanism of action of the fumonisins [18,19]. With the availability of increased quantities of pure fumonisins, several subchronic toxicity studies, designed to establish dose response characteristics in rodents have now been completed, both in our labs and elsewhere. FB_1 was found to be carcinogenic in the rat, although there are shortcomings in the study [20]. It was

not found to be genotoxic. Studies with radiolabelled FB_1 in pigs suggest an accumulation of residues in liver and kidney. The liver has been found to be a target in most species, and the extent of damage is dose, duration and species specific. In many species (pig, rodent) the kidney is also involved. Since FB_1 has affected several target tissues in animal studies, it is difficult to predict what the major target tissues will be in humans. However, since concerns about the chronic toxicity of the fumonisins have not yet been adequately addressed, a TDI cannot be established at this time. With the information at hand it is, nevertheless, possible to arrive at an interim risk assessment, which can be used to make interim risk management decisions. In addition to the results with pure fumonisins, one needs to consider that naturally *F. moniliforme*-contaminated food contains other contaminants besides FB_1, such as other fumonisins and other toxic *F. moniliforme* metabolites.

Because of the ubiquitous nature of *F. moniliforme*, a 5-year monitoring survey for the presence of FB_1 and fumonisin B_2 (FB_2) in Canadian corn and corn-based food items is being conducted by the HPB. Preliminary results of the survey were recently published [8]. The mean FB_1 content was calculated by assuming that the level of FB_1 in samples below the detection limit was either equal to zero (ND = 0), or equal to the detection limit (ND = DL). The true mean is somewhere between these 2 estimates. To the FB_1 level, a value of 20% was added for FB_2, based on the overall ratio of the levels of FB_1 and FB_2 typically seen here and elsewhere. The dried corn/corn meal/corn flour commodities had the highest levels of fumonisins, which averaged 0.20 and 0.28 μg/g for ND = 0 and ND = DL, respectively. Since fumonisins are relatively heat-stable, it was assumed that there would be no loss of fumonisins either during processing or cooking of fresh or dried corn. Corn and corn product consumption rates were obtained in most instances from the 1970–72 Nutrition Canada Survey [21]. For some food commodities this information was not known, and either other sources were utilized or some assumptions were made regarding the intake of a given commodity, usually by comparing it to the intake of a similar type of food, for which consumption data were available. In other instances, the product analyzed would not be consumed as such (e.g. corn meal, corn flour), and estimated intakes of the corn portion of the finished product were made. The 'all person' intake, which is the intake estimate from several corn-based foods at an average rate for all persons, was made to address possible concerns about a chronic intake of fumonisins. For 5–11-year-old children the estimates ranged from 0.025 to 0.089 μg/kg body wt./day, with the other age categories consuming less than this. The maximum estimate is still considered conservative, since it would likely decrease if detection limits were to be less than 0.1 μg/g.

As an alternative to setting a preliminary TDI, the observed Canadian fumonisin exposure estimates can be compared to the effects seen in animals. For chronic effects the maximum 'all person' intake by 5–11-year-old children of 0.089 μg/kg body wt./day is:

- 1100 times less than the NOAEL for the induction of field cases of a fatal disease in horses;
- about 2400 times less than the NOAEL of 0.21 mg/kg body wt./day in a 90-day rat study; this study does not address possible carcinogenic effects because of its relative short duration of exposure;
- 1900 times less than the NOAEL of 0.17 mg/kg body wt./day of FB_1 and FB_2 in the 14-day pig study; similar to the above rat study; this study does not address possible carcinogenic effects because of its short duration of exposure, and very few animals were studied;
- 1700 times less than the NOAEL of 0.15 mg/kg body wt./day in the chronic Vervet monkey study; with so few animals in the study, although of long duration, the carcinogenic potential in this species cannot be addressed; and
- 42 000 times less than the dose of 3.76 mg/kg body wt./day in the 26-month BDIX rat study, which induced liver cancer in 66% of the animals tested, as well as liver nodules

and liver foci. This study suffered from a number of insufficiencies, such as few animals studied and only 1 dose level.

Based on these comparisons, and other information on the fumonisins, it may be concluded that the intake of FB_1 and FB_2, for the most sensitive age group, is unlikely to pose a health hazard.

Experimental carcinogenicity studies with the fumonisins have been hampered by a lack of pure toxins. Major efforts are now ongoing in Canada, USA and South Africa to produce fumonisins on a large scale. Further carcinogenicity studies are being conducted in South Africa (using lower dosage levels) and by the US Department of Health and Human Services National Toxicology Program. Further epidemiological studies are required to more precisely define the role of *F. moniliforme* and its metabolites in the development of esophageal cancer in Transkei and other areas where the incidence of esophageal cancer is high, such as parts of China and northern Italy. In several of these areas there is a high incidence of *F. moniliforme* in corn, high levels of fumonisins in corn, and the per capita corn consumption often is much higher than in Canada.

There is a need to continue with the surveys of fumonisins in corn-based food products being marketed in Canada, because of year-to-year climatic fluctuations.

4. Conclusion

The low levels of fumonisins found to date in corn-based foods in Canada do not appear to pose a human health risk, based on a comparison with effects seen at much higher levels of fumonisins in various animal species. There appears, therefore, to be no need to establish guidelines or action levels for fumonisins in corn-based foods in Canada at the present time. The current regulations for aflatoxins in nut and nut products have been useful in ensuring that the exposure to AFB_1 from nuts and nut products in Canada is one of the lowest in the world, and is unlikely to present a health risk in the absence of HBV infection.

Some of both the fumonisins and aflatoxins produce liver foci, hyperplastic nodules and eventually hepatocarcinogenesis in rats, with those in the former group appearing to be non-genotoxic and those in the latter group being potent genotoxic agents. More information on the mechanism of hepatocarcinogenesis and the significance of the various biomarkers for the classification of foci may be useful to better understand underlying differences in the mechanism of action of these 2 groups of substances and their relative role in the initiation, promotion and progression of liver and possibly other cancers. Such information ultimately will provide a better basis for risk assessments.

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Genetic implications in the metabolism and toxicity of mycotoxins

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Abstract

In common with many xenobiotics, metabolic activation and detoxification play crucial roles in the determination of a toxic response of animal species, including man, to exposure to mycotoxins. Control of expression of the relevant enzymes, both constitutive and inducible, is therefore a major factor in mycotoxin-induced acute or chronic toxicities. The involvement of these factors in the toxic responses to aflatoxins and ochratoxins will be briefly reviewed. In the case of the aflatoxins, the importance of secondary, conjugating metabolism has become increasingly evident. The specific control of expression of these enzymes, through sequences present in the 5′ region of the gene, is evident (e.g. by the changes seen during development and differences between the sexes). The existence of these control mechanisms has made feasible the development of chemoprotective strategies. Although less detailed information is available concerning the metabolic activation and detoxification of the ochratoxins, it appears probable that future studies will reveal a role for the genetic control of expression of enzymes responsible for the target nephrotoxicity of these mycotoxins.

Keywords: Mycotoxins; Aflatoxin B_1; Ochratoxin A; Liver cancer; Epoxide; Cytochrome P450

The mycotoxins comprise a large and structurally diverse group of toxins, having in common their origin as secondary metabolites produced by fungi. There is evidence indicating that many mycotoxins require metabolic activation in order to induce a toxic response. This can be demonstrated by a lack of a significant toxic response in vitro, when test organisms (e.g. mammalian cells), lacking metabolising capacity, are exposed to the mycotoxins in the absence or presence of added metabolising systems. Since the expression of many mammalian xenobiotic metabolising systems are known to be under complex genetic control, there is a need for detailed information on these aspects in order to make assessments of the potential risk to humans from exposure to these toxins.

One group of mycotoxins, the trichothecenes, on the basis of in vitro tests, appear to possess directly toxic properties. This could be associated with the presence of an epoxide group.

Although primary metabolic activation might not be required for these compounds to exert a toxic effect, there remains the possibility of a genetic influence on their toxicity through the level of expression of secondary detoxifying metabolism. Recent studies have demonstrated the importance of secondary, conjugative metabolism in determining the extent of toxic responses to exposure to many toxins. In common

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with the majority of xenobiotics the biological response to mycotoxins, requiring metabolic activation, depends on the balance between activating and detoxifying metabolism. Present knowledge of the metabolism of most of the mycotoxins is not sufficient to allow any detailed analyses of these processes, or of any genetic implications involved. Only in the case of the aflatoxins, and to a lesser extent the ochratoxins, are there sufficient data to attempt to evaluate possible influences of genetic factors in determining toxicological endpoints. In the case of aflatoxins, the most biologically active and abundant member of this group of toxins in contaminated foodstuffs is aflatoxin B_1 (AFB_1). The major pathways involved in its metabolism are given in Fig. 2.

In all animal species examined, including man, the liver is the target organ for AFB_1. Ingestion of this material by man is known to be capable of inducing acute poisoning, aflatoxicosis, and is believed to be implicated in the development of primary liver cancer.

In the rat, the activation of AFB_1 to AFB_1-8,9-epoxide is principally dependent on CYP 2C, although CYP 1A2 may also have some activity [1]. Induction by phenobarbitone (PB) in vivo increases the level of epoxidation, observed in vitro and also the formation of AFQ_1 in rats [2]. CYP 3A has been found to be active in the production of AFQ_1. The induction of CYP 1A1 by the administration of 3-methyl cholanthrene (3MC) in vivo leads to an enhanced production of the hydroxylated metabolite AFM_1 in vitro [2]. Because these inductions alter the balance

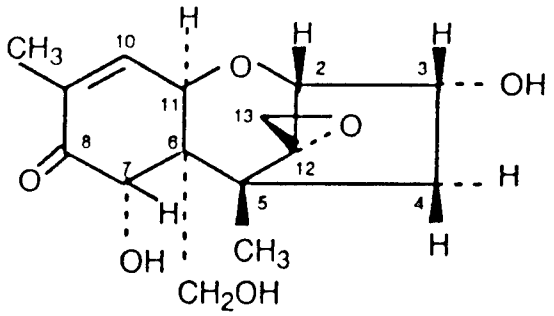


Fig. 1. Structure of deoxynivalenol.

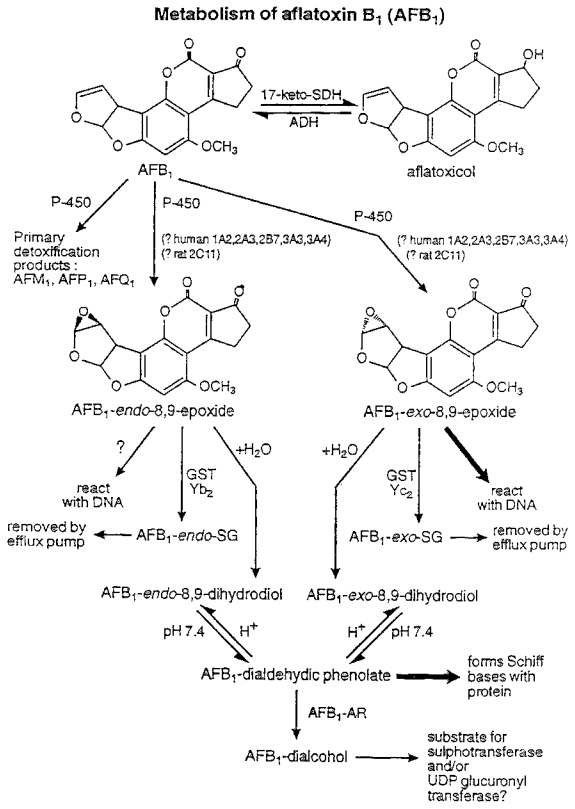


Fig. 2. Metabolism of AFB_1.

between the formation of active and detoxified primary metabolites, they have implications in terms of an effect on the toxic response, modifying that which results from the different levels of constitutive expression of the various cytochromes P450 (e.g. in different strains of rat). A further complicating factor at the level of primary activation of AFB_1 to the AFB_1-8,9-epoxide is the existence of exo and endo isomeric forms of this compound [3]. It has been found that although the exo form undergoes binding to DNA [4], and is the isomer whose conjugation with glutathione (GSH) is catalysed by mouse cytosols [5], both isomers are capable of undergoing conjugation with GSH [6]. In the mouse, virtually 100% of the epoxidation leads to the formation of the exo isomer. In the control rat the exo/endo ratio is 32/1 and in PB-stimulated rat 99/1 showing a greater stimulation of production of the known DNA-binding isomer [3].

There is a possibility that the endo-expoxide may be more stable than the exo which could have implications in terms of the extent of the cytotoxic response. In addition to the effects of PB and 3MC on the primary metabolism of AFB_1, there is now considerable interest in the effects of potential chemoprotective agents, in particular anti-oxidants, on the metabolism of AFB_1. Phenolic anti-oxidants have been found to induce CYP 1A1, resulting in an increased production of AFM_1 [7].

Concerning the primary metabolism of AFB_1 in humans, the reactive metabolite, as in the rat, is the AFB_1-8,9-epoxide. Both endo and exo isomers are produced, with higher proportions of the endo to exo form than in the rat [3]. Interestingly, in the few cases so far examined, there has been found to be a very considerable variation in the exo/endo ratio from 20/1 to 2.8/1 [3]. Clearly genetic control of constitutive levels of expression of the enzymes producing the AFB_1-8,9-epoxide and in particular the relative proportions of the 2 isomers must play a crucial role in determining the individual sensitivities to this toxin. In humans, it appears that more than one cytochrome P450 is involved in the activation of AFB_1. There have been some differences of opinion as to which cytochrome plays the major role. Studies based on inhibitions using isoform-specific antibodies or chemicals have indicated CYP 3A4 as being the predominant isoform involved [8]. Studies using bacterial recombinant human cytochrome P450 enzymes showed that CYP 3A4 catalysed the formation of the greatest amount of epoxide which was entirely the exo isoform, whereas CYP 1A2 produced lower amounts of the AFB_1-8,9-epoxide which also was a mixture of the 2 isomers [9]. Other workers have claimed that the metabolism due to CYP 1A2 is the most relevant in terms of human toxicity since it has a high affinity for AFB_1 and therefore would be active at the low levels of AFB_1 likely to be present in in vivo exposure to the toxin [10]. This view has been supported using human cell lines transfected with CYP 1A2 and CYP 3A4 [11]. Genetic variation in expression of the individual cytochrome P450s producing the AFB_1-8,9-epoxide and in particular these 2 isoforms clearly requires further study in view of their importance in the production of an acutely toxic or carcinogenic response. The possibility that the polymorphic expression of the CYP 2D6 gene in humans might be associated with the development of cancer has received considerable attention. Extensive debrisoquin metabolisers are at an increased risk of developing lung, liver or bladder cancer, and individuals possessing inactivating mutations in the gene were more frequent in cases of primary liver cancer [12]. The suggestion that the expression of this gene might be involved in the activation of AFB_1 have not been demonstrated in vitro [13]. However, studies on AFB_1 hepatocarcinogenesis in the CYP 2D6 deficient DA rat have demonstrated an apparent lower induction of liver cancer in these rats than in the CYP 2D6 positive F344 rat (Neal and Idle, unpublished observation). It appears possible that the CYP 2D6 polymorphism affects AFB_1 hepatocarcinogenesis by a mechanism other than at the level of production of AFB_1-epoxide. A further factor which may affect the human response to AFB_1 at the level of primary metabolism is the preferential induction of detoxifying pathways, thus reducing the amount of toxin available as a substrate for activating metabolism. This appears to have parallels to the protective effect of 3MC on AFB_1 toxicity in the rat. It has been reported that in the Fujian province of China, an area of heavy contamination with AFB_1, a higher incidence of primary liver cancer was noted amongst non-smokers compared with smokers [14]. It appears probable that the polycyclic aromatic hydrocarbon content of cigarette smoke induced the expression of CYP 1A1-mediated metabolism of AFB_1 to AFM_1, in a way analogous to 3MC induction in the rat.

Another genetic factor at the level of primary metabolism of AFB_1 which is possibly involved in AFB_1-induced liver cancer in humans is the control of expression of the cytochrome P450 catalysing the *O*-demethylation of AFB_1 to AFP_1. The presence of AFP_1 in urine has been found to be correlated with the development of primary liver cancer, and in in vitro studies AFP_1 formation from AFB_1 was highly elevated in

microsomal fractions isolated from tumour tissue compared with control tissue [15]. There is little information in the literature concerning the cytochrome P450 catalysing this reaction.

It is becoming increasingly evident that a highly significant role in AFB_1 toxicity is played by the secondary, conjugating metabolism [16]. Glucuronidation, sulphation, and acetylation of primary AFB_1 metabolites are likely metabolic processes, but the substrates for these conjugations would be hydroxylated primary AFB_1 metabolites (e.g. AFM_1, AFP_1 and AFQ_1) whose toxic and carcinogenic potentials are much lower than the parent AFB_1. These conjugating reactions therefore probably play a minor role in the detoxification process. The major conjugative process, with a crucial influence on the toxic outcome of metabolism, is the formation of a GSH conjugate from AFB_1-8,9-epoxide. This reaction is primarily catalysed by glutathione *S*-transferase enzymes of the α class in the case of the exo-epoxide, whereas endo-epoxides are better substrates for the μ class enzymes [6]. The high activity in mouse liver cytosol exclusively conjugates the exo isomer, whereas the low activity in rat cytosol conjugates both isomers with a preference for the endo form [6]. The μ class enzymes are polymorphic in humans, which has raised the possibility of genetic control of sensitivity to the toxins at this level. However, since AFB_1-DNA adducts appear to be predominantly, if not exclusively, formed from the exo-epoxide, the interaction of the endo-epoxide with DNA may have little if any effect on genotoxicity, but the possibility remains of an effect on cytotoxicity. Modification of interactions of the endo-epoxide with RNA and protein could result from conjugation by μ class enzymes. Protein binding of AFB_1 takes place via AFB_1-dihydrodiol and not the epoxide, so a slower rate of hydrolysis of the endo-epoxide to form a dihydrodiol could facilitate a wider spread of cellular damage than that resulting from the dihydrodiol formed from the exo-epoxide. In the rat, conjugation of the exo-epoxide to form the GSH conjugate is catalysed by members of the α class GST containing the Yc subunit, and in particular the Yc_2 subunit. Genetic control of expression of the Yc_2-containing enzymes includes high expression in the neonate, very low expression in the adult with a higher expression in females than males. Inducer-dependent expression results from the dietary administration of a range of compounds, including synthetic and naturally occurring anti-oxidants. Elevation of Yc_2-containing GSTs correlates with the chemoprotection against the induction, by AFB_1, of pre-neoplastic lesions in the liver. Likely genetic control mechanisms involve the anti-oxidant response element (ARE) in the 5′ region of the gene, but other control elements also appear possible [17]. The Yc subunit, highly homologous to the Yc_2 subunit in the rat, is constitutively expressed in the mouse. This demonstrates a fundamental difference in genetic control of expression of this GST subunit between rat and mouse, and underlies the differing species sensitivity to AFB_1 toxicity [16]. In humans, as referred to above, conjugation of the endo-epoxide of AFB_1 probably proceeds via the μ class GST, whose expression is polymorphic. The α class GSTs, constitutively suppressed in human liver cytosol, have little conjugating activity towards the genotoxic exo-epoxide [18]. Chemoprevention strategies against AFB_1 genotoxicity in humans, involving the administration of anti-oxidants such as the drug oltipraz are based on a presumed capacity for the induction of GSTs, homologous to the Yc subunit constitutively expressed in the mouse and inducible in the rat [13]. To date, evidence for this capacity is limited. Another enzyme with potential for protection against AFB_1 toxicity which has recently been investigated, catalyses the reduction of AFB_1- dihydrodiol to the dialcohol. This enzyme, a member of a family of aldehyde reductases, converts a metabolite of AFB_1, AFB_1-dihydrodiol, which is believed to be the major protein binding form of the toxin and which has implications in terms of the cytotoxic response, into a non-binding form [19]. The dialcohol then probably undergoes conjugation and is excreted. The AFB_1-dihydrodiol aldehyde reductase (AFAR) is expressed to a very low level in the adult rat liver, but is capable of inducer-dependent expression in a very similar manner, and in

response to the same inducing substances, as the Yc_2 subunit of rat liver GST. The induction of the expression of this enzyme in humans in response to anti-oxidants would clearly have implications in terms of chemoprotection. As in the case of the GST Yc_2 subunit, the inducer-dependent expression of these enzymes in the rat is probably dependent on responsive element sequences in the 5′ region of the gene.

In summary, the sensitivity of the rat to AFB_1 toxicity is under genetic control, involving the expression of individual cytochrome P450s and the secondary GSH conjugating and aldehyde reductase enzymes. The constitutive expression of these enzymes can be modified by the action of inducing substances on controlling regions in the genome, with profound effects on the toxic response to AFB_1.

In the case of ochratoxin A (OTA), the metabolic pathways given in Fig. 3 have been defined. The inhibition of protein synthesis, resulting from the administration of the compound to rats and mice has been shown to be due to an inhibition of phenylalanine-tRNA formation [20].

A general inhibition of protein synthesis would appear unlikely to account for the toxin targeting the kidney in both animals and man. In the case of animals, the toxin has been demonstrated to produce kidney tumours in rats and mice, and there is strong evidence for a nephrotoxic effect in pigs. In the case of humans, exposure to the toxin has been suggested to be implicated in the disease termed Balkan endemic nephropathy (BEN) [21].

Concerning the metabolic aspects of the toxicity of OTA, the toxin was reported to be negative in the Ames test and not to bind to DNA. However, other studies using culture medium plus OTA, following conditioning by rat hepatocytes, induced mutations in the Ames test and sister chromatid exchange in human peripheral

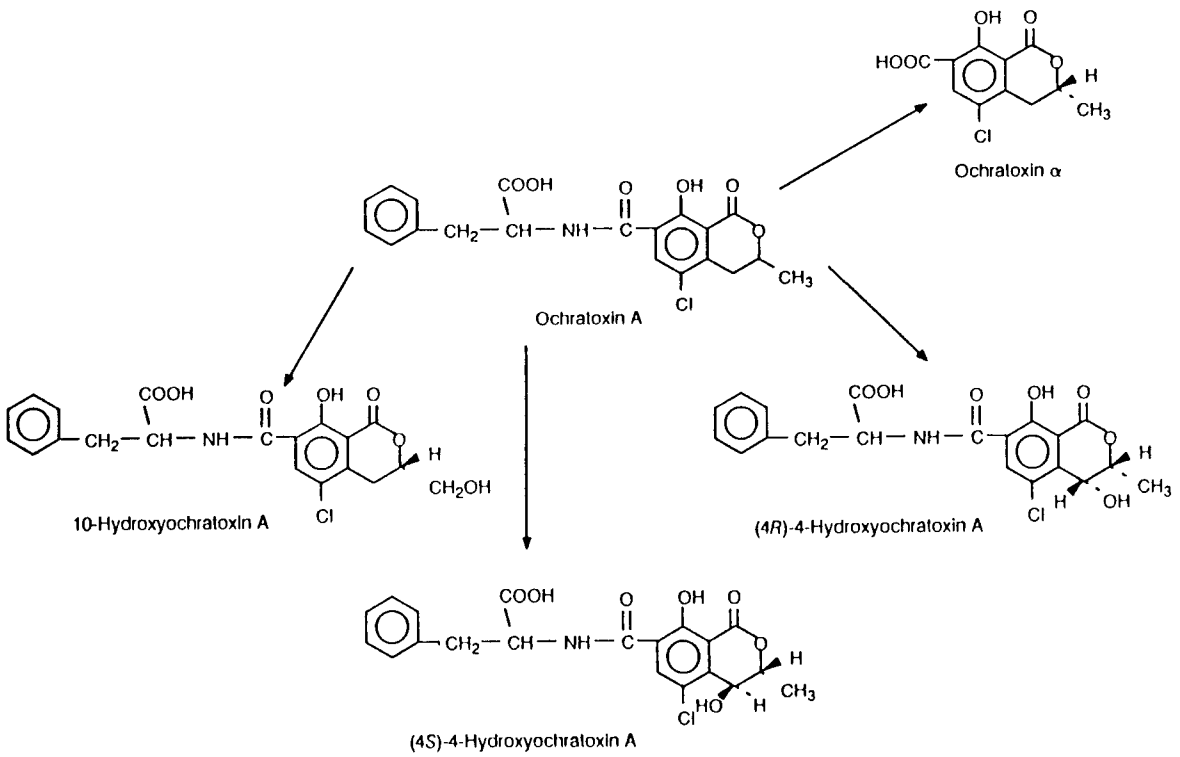


Fig. 3. Metabolism of OTA

lymphocytes in vitro. These results indicate a genotoxic potential for OTA, which has been supported by the induction of SOS DNA repair in *E. coli*. Also other studies have demonstrated that the prolonged feeding of rats with low levels of OTA results in single-strand breaks in liver and kidney DNA [22]. Using [P^{32}] post-labelling techniques, the presence of adducts in the DNA of kidney, liver and spleen (OTA is immunosuppressive) has been detected following the administration of OTA to mice. The adducts differed qualitatively and quantitatively between the 3 organs, there being a higher level of adducts in the kidney which persisted longer than those in the other 2 organs [23]. All of these data identify OTA as a genotoxic agent. However, regarding the metabolic activation of the toxin, little is known at present. The formation of different adducts in the liver and kidney suggests different metabolic routes of activation. It has been demonstrated in *E. coli* that β-lyase action on a cysteinyl-OTA conjugate is probably associated with the cytotoxic reaction [24]. Kidney toxicity from certain chemicals (e.g. hexachlorobutadiene) has been found to result from a β-lyase cleavage of cysteinyl conjugates. This indicates a possible biochemical mechanism for the nephrotoxic action of OTA. The genetic control of expression of both the primary activation and conjugation of OTA with GSH and the subsequent reactivation by β-lyase could play a role in the toxicity of OTA.

With respect to the primary metabolism of OTA, similar to the case of the aflatoxins, parallels have been drawn between this activity and the oxidation of debrisoquin. Animal species differing in their capacity to oxidise debrisoquin, also differ in their capacity to form hydroxylated metabolites of OTA. The DA rat, which is a poor metaboliser of debrisoquin, is also a poor metaboliser of OTA. Antibody and induction studies, however, have indicated that the cytochrome responsible for OTA hydroxylation is not identical with CYP 2D6, but may also share some epitopes with CYP 1A1. The possible relevance of these metabolic studies to man is indicated by the results of a study which found that a group of BEN patients contained a significantly higher proportion of rapid debrisoquin oxidisers, than did non-BEN individuals from the same area [25].

In summary, there therefore appears to be an involvement of metabolism, both activating and detoxifying in the nephrotoxic action of OTA. Further research is necessary to define the metabolic pathways involved and to evaluate the genetic control of their expression, both constitutive and inducible. Hopefully, on the basis of the results of these studies protective strategies will be devised, to complement the reduction in OTA-induced pathology, due to a lowering of human exposure to this mycotoxin.

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Prevention of nephrotoxicity of ochratoxin A, a food contaminant

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by ubiquitous *Aspergilli*, mainly by *Aspergillus ochraceus* and also by *Penicilium verrucosum*. It is found all over the world in feed and human food and blood as well as in animal blood and tissues. The most threatening effects of OTA are its nephrotoxicity and carcinogenicity, since this mycotoxin is nephrotoxic to all animal species studied so far and is increasingly involved in the Balkan endemic nephropathy (BEN), a human chronic interstitial nephropathy which is most of the time associated to urinary tract tumours. Since it seems impossible to avoid contamination of foodstuffs by toxigenic fungi, detoxification and detoxication for OTA are needed. To reduce or abolish the OTA-induced toxic effects, several mechanisms were investigated. The results of these investigations showed that some of the potential antidotes were efficient in preventing the main OTA toxic effects whereas some others were not. Promising compounds are structural analogues of OTA, and/or compounds having a high binding affinity for plasma proteins such as piroxicam, a non-steroidal anti-inflammatory drug (NSAID). Some enzymes such as superoxide dismutase (SOD) and catalase, radical scavengers, vitamins, prostaglandin (PG) synthesis inhibitors, (such as piroxicam), pH modificators, adsorbant resin such as cholestyramine etc. are efficient in vivo. Some of the results obtained in vivo were already confirmed in vitro and gave useful information on how to safely use these antidotes. The most generally acting compound seems to be A19 (Aspartame®), a structural analogue of OTA and phenylalanine. When given to rats A19 (25 mg/kg/48 h) combined to OTA (289 μg/kg/48 h) for several weeks largely prevented OTA nephrotoxicity and genotoxicity. When given after intoxication of animals with OTA it washes out the toxin efficiently from the body. In vitro, A19 (10 μg/ml) prevents OTA (20–500 μg/ml) binding to plasma proteins. Its general action without any known side effect in humans and in animals, points at A19 to be the best candidate for preventing the OTA-induced subchronic effects.

Keywords: Ochratoxin A; Aspartame; Nephrotoxicity; Genotoxicity; Phenylalanine

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by fungi of *Aspergillus* and *Penicillium* genera [1]. It is a food contaminant found not only in cereals (wheat, maize, barley) but also in beans, dried fruits, coffee, cocoa. It is also found in human blood, urine and milk as well as in animal blood and tissues [2–7]. In many Balkan areas, people suffering from chronic interstitial nephropathy and/or urinary tract tumours have high

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concentrations (2–40 ng/ml) of OTA in their blood as well as in their food (1–35 μg/kg).

The importance of OTA in the public health and economy is becoming increasingly evident because of its implication in Balkan endemic nephropathy (BEN) and also because it has been found in food and human blood in Germany and other western European countries including France, Italy, Scandinavia, and also Canada and Japan [8–13].

Since OTA was found in human and animal blood and food in Northern Africa, Tunisia, Algeria and Egypt [14] associated with many cases of nephropathy [15] the ochratoxin contamination should be regarded from now on as a worldwide problem.

When introduced in the body, OTA binds strongly to plasma proteins and is metabolised into hydroxylated compounds, one of which, the (4*R*)-4-hydroxyochratoxin A being as cytotoxic and immunosuppressive as the parent compound in vivo and in vitro [16,17]. OTA can be cleaved into phenylalanine (Phe) and OTα, the chlorinated dihydroisocoumarinic moiety found not to be toxic, but retaining some genotoxicity [18].

The toxicological aspect of OTA contamination becomes more complex since natural analogues of the toxin were found in *Aspergillus ochraceus* no. 589-68 NRRL culture on grains [19] and since OTα considered so far as non-toxic retains genotoxic properties [18]. Acute and chronic toxicity of OTA are related directly or indirectly to its property of inhibiting protein synthesis by competition with Phe in the reaction catalysed by phenylalanyl-tRNA synthetase [20–23]. OTA is also able to inhibit all the reactions in which Phe, its structural analogue, is involved, such as those catalysed by Phe hydroxylase [24]. The same considerations apply to all those reactions involving the amino acid structural analogues of OTA such as tyrosine hydroxylase leading to DOPA for tyrosine-OTA which is cytotoxic [17].

Toxic effects of ochratoxins are also related at least partially to the oxidative processes, to the intracellular handling of calcium [25], to the inhibition of mitochondrial respiration and ATP production [26,27]. All these reactions result from the inhibition of protein synthesis.

This paper updates this research and focuses on the beneficial effects of A19, a structural analogue of both Phe and OTA, on the nephrotoxicity and genotoxicity induced by subchronic exposure to the toxin.

2. Mechanism of action

OTA is a structural analogue of L-β-Phe with a 7-dihydroisocoumarin chlorinated in position 5 [1,28]. The chlorine atom seems to play a key role due to electronegativity because the dechloro derivative (OTB) is much less cytotoxic or not toxic at all [29].

2.1. Inhibition of protein synthesis

OTA inhibits protein synthesis in vivo and in vitro at the elongation step by inhibiting the binding of Phe to its specific t-RNA [21–23,30–32]. This mechanism has been confirmed by the synthesis of analogues of OTA possessing other amino acids instead of Phe. They inhibit the reactions catalysed by the specific aminoacyl-tRNA synthetases appropriate to the amino acid replacing Phe in their structure [17].

In the meantime natural analogues have been isolated by Hadidane et al. [19] indicating that in the case of natural contaminations, humans and animals are exposed to a mixture of toxins with consequent synergistic effects. It is well known that natural contamination by OTA-producing fungi is more potent in inducing porcine nephropathy than is the artificially OTA-contaminated feed for the same OTA levels [2,33,34].

These inhibitory properties of OTA, which explain almost all the toxic effects of OTA, include effects on certain enzymes involved in its metabolism, cytochrome P450 (P450), aminopyrine demethylase and alanine hydroxylase levels are all reduced by OTA at doses of about 1.5 mg/kg/15 days [35].

Phosphoenol pyruvate carboxykinase activity is also reduced in the kidney [36,37].

2.2. Implication of oxidative pathways

Rahimtula et al. [38] have shown that OTA induces lipid peroxidation in the presence of

microsomes and NADPH and traces of chelated Fe^{3+}. This lipid peroxidation is determined, in vitro, by malonaldehyde formation and, in vivo, by hexane exhalation. The first consequence of this harmful mechanism is the modification of membrane permeability and, consequently, cellular necrosis.

The production of free radicals is generally prevented by antioxidant and radical scavengers such as vitamin E, vitamin A, and superoxide dismutase (SOD) combined with catalase. The other oxidative pathways implicated in the metabolism of OTA are related to the cooxidation during prostaglandin (PG) synthesis. For the prevention of these reactions, inhibitors of PG synthesis and of cyclooxygenase could be used. Some of these inhibitors only partially block this pathway, others can block completely the whole system, for example piroxicam [39].

3. General metabolism

3.1. Production of OTα

OTA is cleaved into Phe and OTα by peptidases in the intestinal mucosa, and in other organs such as pancreas as well as in vitro. OTα was previously regarded as harmless, but evidence for its genotoxicity have been recently found [18]. Thus the level of OTα should also be determined in humans and in bigastric animals to assess the exposure.

3.2. Production of hydroxylated derivatives

In the presence of NADPH, liver microsomes from pig, rat and humans metabolise OTA to produce hydroxylated derivatives (4*R*)- and (4*S*)-4-hydroxyochratoxin A [40–42]. In the same conditions rabbit liver microsomes produce 10-hydroxyochratoxin A [43].

OTA is also used as a substrate by Phe hydroxylase and transformed into tyrosine OTA (Tyr-OTA) [24]. This derivative is cytotoxic [30].

The P450 involved in the hydroxylation were, for the rat, CYP1A2 for the (4*R*)-isomer and CYP2B1 for the (4*S*)-isomer. In the pig microsomes the implicated cytochromes were CYP2B and CYP2C11 classified A2 and A3. One OTA metabolite more lipophilic than the parent compound has also been identified in pig liver microsomal incubations [42].

The implication of OTA metabolism in its nephrotoxicity has been analysed by Delacruz and Bach [44].

4. Structural analogues of OTA and related compounds (Phe and piroxicam)

Phe and piroxicam favour the absorption of OTA from the stomach but seem to prevent it from being distributed to all organs. In using these compounds the blood-OTA concentration was effectively higher but the intestinal absorption decreased. Urinary elimination was enhanced by 70 and 110% for Phe and piroxicam, respectively, within the first 24 h. In normal conditions and after dosing rats for 2–6 weeks with 289 μg/kg/48 h of OTA, the blood contained 3.3–3.6 μg/ml while the urine contained 0.13–0.24 μg/ml [45].

Phe influences the toxicokinetics of OTA. It increases gastrointestinal absorption, intestinal transit, urinary and intestinal elimination. The half-life time is reduced, and the acute toxicity is largely prevented [31,46–49].

It should be remembered that Phe has no effect at all on the genotoxicity of OTA [48], indicating that the reactive compounds responsible for this genotoxicity are not structurally related to Phe. This includes Tyr-OTA, OTα and perhaps conjugates of OTA as candidates for these harmful chronic effects.

Phe is very efficient on protein synthesis inhibition induced by OTA [16,30,31,46].

5. A19 as detoxicating agent

5.1. Structural analogy and specific metabolism in animal and humans

A19 is Aspartame, i.e. the methyl ester of aspartyl Phe. It is thus a structural analogue of both Phe and OTA. Comparing the metabolism in humans and animals Ranney et al. [50] showed

that similarly in both species A19 was completely cleaved into aspartate, Phe and methanol within a few hours. They have found that the major portion of the [^{14}C] label was expired (10–24%) within 24 h while 0.7–11% was found in faeces in urine and about 10% was incorporated into proteins. The plasma peak occurred after 4–7 h.

Recently, Moller [51] reported a very meaningful study on A19 metabolism in humans. Six human males each received 0.56 g Phe in the form of 1.0 g aspartame or 12.2 g bovine albumin in 200 ml water or water alone. Venous blood samples collected before consumption and during the following 4 h were assayed for plasma levels of large, neutral amino acids (LNAA), aspartate, insulin and glucose. The area under the curve for plasma Phe was 40% greater, although not significant, after aspartame compared with albumin intake. The indicated increased clearance rate of plasma Phe following the intake of albumin may be caused by a significant increase in insulin, on which aspartame had no effect. There was a significant effect of aspartame on plasma tyrosine but not on tryptophan, valine, isoleucine or leucine. Plasma aspartate was significantly increased at 0.25 h after the aspartame intake. The percentage Phe/LNAA decreased slightly in response to albumin but increased 55% after aspartame and remained significantly increased for 2 h. Tyrosine/LNAA increased and tryptophan/LNAA decreased modestly after aspartame intake. The study showed that the intake of aspartame at a not unrealistically high level produced a marked and persistent increase of the availability of Phe to the brain, which was not observed after protein intake. The study indicated, furthermore, that Phe was cleared faster from the plasma after consumption of protein compared with aspartame.

Because the tissue distribution of the peptide and its elimination were still uncertain, a sensitive detection method for A19 using HPLC and fluorescence after derivatisation with *ortho*-phtalaldehyde had been recently developed (unpublished results). This permits follow-up of the detoxicating agent in body fluids and organs. Several studies have been conducted to determine the protective effect of A19 in nephrotoxicity and genotoxicity according to the methodology described by Baudrimont et al. [52–54].

The results showed that 10–12% of A19 was distributed unchanged in blood urine and organs (kidney, liver, brain and testicles). When given alone to rat, A19 (25 mg/kg) resulted in levels of 73 ± 6, 1.8 ± 0.1, 156 ± 9, 34 ± 2 μg/g, 66 ± 5, 19 ± 2 μg/ml in kidney, liver, brain, testicle, serum and urine, respectively. In the presence of OTA (289 μg/kg) the levels were as follows for the same organs and fluids, 68 ± 5, 2.1 ± 0.1, 105 ± 9, 25 ± 0.6 μg/g, 45 ± 3 and 11 ± 0.2 μg/ml. The presence of μg quantities of A19 had not been reported before due to the sensitivity of the methods used previously in the 1970s. These quantities of A19, however, seem extremely important for its mechanism of antagonism in OTA binding to human plasma proteins, transport, elimination, metabolism etc.

5.2. Effect of A19 on OTA binding to human plasma proteins

At a comparable blood concentration (10 μg/ml) the effects of A19 on OTA binding to human plasma proteins have been investigated in a 2-compartment dialysis system, in the 2 following conditions: OTA was put in the lower compartment containing NaCl 0.9%, pH 7.2, whereas A19 was added to the upper one containing plasma; OTA was added to the lower compartment simultaneously with A19 and the human plasma alone in the upper one. After the indicated time, the solution containing free OTA (NaCl 0.9%), pH7.2, is removed and replaced by a fresh solution without toxin in a new run to avoid the presence of free OTA in the other side. So only bound OTA was assayed by HPLC and fluorescent detection.

The results are summarised in Tables 1 and 2. Ochratoxin concentrations used in these experiments were very high, exceeding those found in natural contaminations. They have been put intentionally to show the very high efficacy of A19 to prevent the OTA binding on human

Table 1
Effects of A19* (10 μg/ml) on the binding of OTA to human plasma proteins in vitro, checked by dialysis, through a membrane having an exclusion limit at 10 kDa

OTA concentrations (μg/ml)	Time (min)	OTA bound	
		OTA alone (ng/ml)	OTA + A_{19} (ng/ml)
20	30	215 ± 11	157 ± 12
	60	431 ± 18	97 ± 4
	120	772 ± 25	15 ± 3
100	30	442 ± 21	193 ± 17
	60	739 ± 17	74 ± 11
	120	1231 ± 71	55 ± 15
500	30	556 ± 15	275 ± 41
	60	1175 ± 71	296 ± 45
	120	1744 ± 112	345 ± 51

*A19 was added to the plasma in the upper compartment before OTA was added to the lower one.

Table 2
Effects of A19* (10 μg/ml) on the binding of OTA to human plasma proteins in vitro, checked by dialysis, through a membrane having an exclusion limit at 10 Da

OTA concentrations (μg/ml)	Time (min)	OTA bound	
		OTA alone (ng/ml)	OTA + A19 (ng/ml)
20	30	163 ± 42	159 ± 13
	60	488 ± 41	53 ± 6
	120	774 ± 43	39 ± 4
100	30	491 ± 39	245 ± 15
	60	983 ± 48	459 ± 27
	120	1296 ± 61	587 ± 38
500	30	539 ± 41	516 ± 19
	60	1347 ± 92	1127 ± 89
	120	1899 ± 89	1789 ± 56

*A19 was placed in the lower compartment with OTA.

plasma proteins. The efficacy was higher when A19 was present in plasma prior to OTA and decreased when the toxin concentrations increased. A19 was also capable of displacing OTA from blood plasma proteins.

5.3. Effects of A19 on OTA-induced nephrotoxicity in rats

Several parameters have been investigated to evaluate the nephrotoxicity of OTA and the protective effects of A19. These were enzymuria, proteinuria, glucosuria. In all cases beneficial effects of A19 have been observed. An example is given in Table 3 for enzymuria.

5.4. Effect of A19 on OTA elimination from kidney

The efficacy of A19 to eliminate OTA from the body and mainly from the kidney has been investigated. One group of animals treated for 6 weeks with OTA (289 μg/kg/48 h) was afterwards treated with A19 (25 mg/kg/48 h) and compared to those treated with OTA alone. The results showed lower blood and kidney tissue

Table 3
Influence of A19 on the variations of enzymatic activities in urine of rats treated for 6 weeks with OTA (289 μg/kg/48 h) combined with A19 (25 μg/kg/48 h)

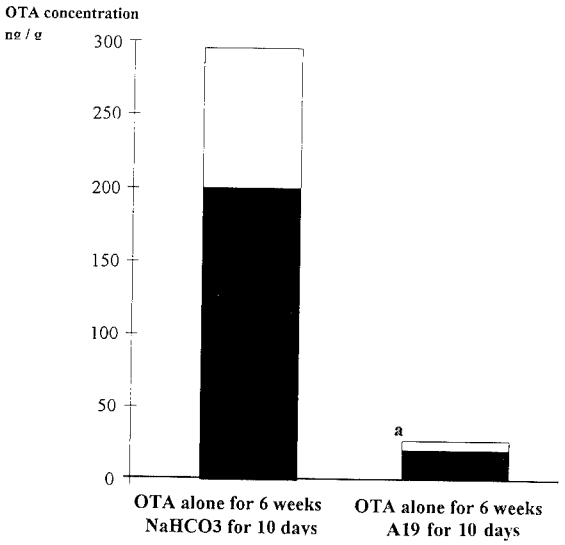
Animal groups	γGT		LDH		LAP		PAL	
	Activity (IU/l)	% Increase	Activity (IU/l)	% Increase	Activity (IU/l)	% Increase	Activity (IU/l)	% Increase
Controls	19 ± 4	–	15 ± 2	–	2 ± 0.4	–	25 ± 5	–
$NaHCO_3$	19 ± 3	–	17 ± 3	–	3 ± 0.5	–	29 ± 4	–
OTA	33 ± 6*	73	28 ± 8***	64	5 ± 1.1*	66	72 ± 8*	148
OTA + A19	23 ± 3**,****	21	22 ± 2**,*****	29	4 ± 0.7**,*****	33	48 ± 6*,****	65

A19 alone does not induce any change.
* Significantly different from animals treated with $NaHCO_3$, $P < 0.01$.
** Not significantly different from animals treated with $NaHCO_3$, $P < 0.01$.
*** Not significantly different from animals treated with $NaHCO_3$, $P < 0.01$.
**** Significantly different from animals treated with OTA alone, $P < 0.01$.
***** Not significantly different from animals treated with OTA alone, $P < 0.01$.

Table 4
Attempt of curative treatment with A19

	OTA concentrations (μg/ml)	
	Day 0 of curative treatment	Day 10 of curative treatment
OTA for 6 weeks and $NaHCO_3$ for 10 days	3.61 ± 0.72	1.01 ± 0.34
OTA/6 weeks and A19/10 days		0.48 ± 0.11*

Influence of A19 on the OTA blood levels in rats treated for 6 weeks with the toxin (289 μg/kg/48 h) and then for 10 days with A19 (25 mg/kg).
*Significantly different from animals treated with $NaHCO_3$ ($P < 0.01$).



[a] *Significantely different from OTA - NaHCO3, p < 0.01*

Fig. 1. Attempt of curative treatment with A19. Influence of A19 on the concentration of OTA in the kidney of rats treated for 6 weeks with OTA (289 μg/kg/48 h), then with A19 (25 mg/kg) for 10 days.

concentrations, after 10 days treatment (Table 4, Fig. 1).

6. Proposed mechanism of action of A19

A19 is absorbed from stomach and intestine into the blood both under the unchanged (10–12% of the given dose) and cleaved form (aspartate and Phe mainly).

The cleavage is time dependent. That gives rise to a pool of Phe which is much more slowly cleared from the body than the one provided by food proteins.

The so provided Phe plays most probably a role as detoxicating agent (prevention of protein synthesis inhibition induced by OTA etc.).

The unchanged A19 (10–12% of the given dose) (which is much more than OTA concentrations in the body in case of natural contamination) prevents OTA binding to plasma proteins,

enhances OTA elimination, enhances OTA metabolism, especially to less toxic and genotoxic metabolites.

All these results confirmed decreasing OTA distribution in organs such as kidney, brain, liver and testicles and finally the prevention of OTA-induced nephrotoxicity and genotoxicity.

7. Conclusions

OTA is a concern of public health by the consumption of contaminated food. The kidney is its main target tissue but OTA is distributed in all organs and produces in these tissues many chronic toxic effects, the more significant in terms of danger to health being nephrotoxicity, genotoxicity and carcinogenicity.

Since it seems impossible to prevent toxinogenic fungi from growing and producing mycotoxins and OTA in particular, the emphasis on detoxication of OTA is an important task from both medical and ethical points of view. Among several compounds A19 has been identified as a useful substance, as a structural analogue of both Phe and OTA.

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Toxicology Letters 82/83 (1995) 879–882

Toxicology Letters

Expression of CYP3A7, a human fetus-specific cytochrome P450, in cultured cells and in the hepatocytes of p53-knockout mice

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Abstract

CYP3A7 is a major form of cytochrome P450 in human fetal livers. To elucidate toxicological significance of CYP3A7 in fetal livers, *CYP3A7* cDNA was introduced into Chinese hamster lung (CHL) cells. Transformants carrying the *CYP3A7* gene were more sensitive to mycotoxins than parental CHL cells. In additional studies, we established a hepatocyte cell line from CYP3A7-transgenic/p53-knockout mice. In hepatocyte cells from CYP3A7-transgenic/p53-knockout mice, CYP3A7 mRNA was expressed and the catalytic activity of CYP3A7 protein was detected. The cells are expected to show cytotoxicity to mycotoxins and teratogens. These cell lines provide a valuable panel for studying the fetal toxicities of chemicals in humans.

Keywords: Transgene; Cultured cells; Genetically engineered cells; Transgenic mice

1. Introduction

Cytochrome P450 (P450) plays central roles in the activation and detoxification of a wide variety of chemicals including carcinogens and teratogens. Among molecular forms of P450, CYP3A7 is regarded as unique due to the following properties. First, CYP3A7 is expressed rather specifically in human fetal livers [1], while in general little or no detectable P450 is present in fetal livers of experimental animals. Second, this form of P450 catalyzes the 16α-hydroxylation of dehydroepiandrosterone 3-sulfate, a precursor of estriol known to be excreted in urine of pregnant women [2]. Third, CYP3A7 catalyzes the metabolic activation of mycotoxins as well as some mutagens produced by protein pyrolysis [3,4]. Since the CYP3A7 enzyme is present as a major form of P450 in human fetal livers [5], it is likely to be one of the causal enzymes responsible for carcinogenicity and teratogenicity of chemicals. Research using human fetal liver may facilitate our understanding of the toxicological roles for

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this cytochrome. However, the use of human fetal liver is currently very limited for ethical reasons.

Thus, this study was undertaken to establish cell lines expressing CYP3A7 to further clarify the toxicological significance of this enzyme in fetal liver. We established cell lines expressing CYP3A7 in Chinese hamster lung (CHL) cells and in hepatocyte cells derived from CYP3A7-transgenic/p53-knockout mice.

2. Advantages of genetically engineered cells to estimate the cell toxicity of metabolites produced by human enzymes

In conventional assay systems, in order to assess the cytotoxicity of metabolites produced by human enzymes it was necessary to add crude enzyme sources including human adult or fetal liver homogenates to the media surrounding cultured cells. In such cases, a constant supply of normal human liver samples is needed (Fig. 1). Additionally, active metabolites, which are generally more chemically reactive than the parent compounds, may bind to cell components existing at the surface of cells. Thus, only a small portion of the active metabolites formed are likely to penetrate through the cytoplasmic membranes. If cells expressing human enzymes, particularly human fetal enzymes, can be established, such cells would offer a useful tool for examining the roles of human enzymes in producing cytotoxic metabolites. In this regard, we have established a variety of relevant cell lines.

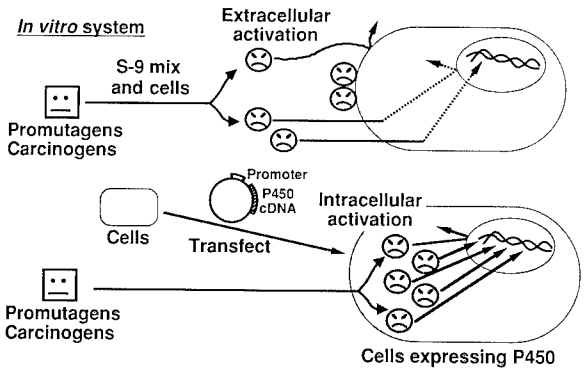


Fig. 1. Metabolic activation of promutagens and toxicological testing.

Another advantage of such systems is that the cells can be supplied constantly without significant changes in the capacity to activate toxicants. In our study, cell lines exhibited similar capacity to activate aflatoxin B_1 even after storage for 2 years. The disadvantage in using such genetically engineered cells may be that the cells express only a limited number of enzymes. If new selection markers are developed in the future, then transformation with increasing number of cDNAs will be possible, resulting in improved and perhaps more accurate estimation of toxicity in humans.

3. Establishment of a CYP3A7 cell line and its application to toxicological testing

Among cell lines we have established, a line expressing CYP3A7 was expected to be a valuable tool since CYP3A7 is expressed specifically in human fetal livers and since aflatoxin B_1 is a known teratogen in human embryos. In our previous studies we demonstrated that CYP3A7 as well as other human CYP3A enzymes could activate aflatoxin B_1 [3,4]. Thus, a plasmid containing the *CYP3A7* or *CYP3A4* cDNA insert downstream of SRα promoter was constructed and transfected to CHL cells. The expression of the CYP3A7 or CYP3A4 mRNA and protein was confirmed by Northern and Western blot analyses, respectively. According to the level of expression, we selected clones 7-40 and 4-10 for CYP3A7 and CYP3A4, respectively, for further experiments. Both clones exhibited higher sensitivity to aflatoxin B_1 as determined by cytotoxicity assay (Fig. 2) [6]. The levels of cytotoxicity of aflatoxin B_1 was nearly the same between the 7-40 and 4-10 cells, indicating that the ability of CYP3A7 and CYP3A4 enzymes to activate aflatoxin B_1 was approximately equivalent. The activation of aflatoxin B_1 by the expressed enzymes was confirmed by the following experiments. First, the effects of addition to the culture medium of α-naphthoflavone (α-NF), a known enhancer of CYP3A, was examined. If the

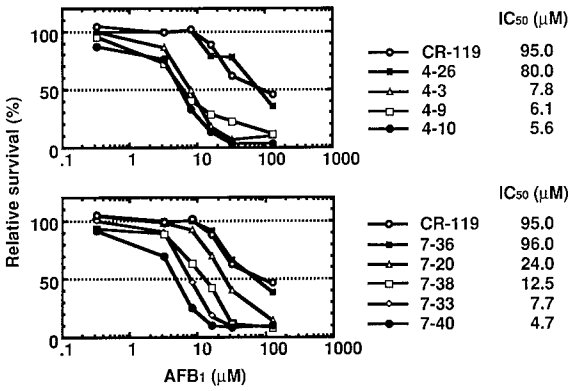


Fig. 2. Cytotoxicity of AFB1 in cells transfected with CYP3A4 or CYP3A7 expression plasmids.

CYP3A enzymes expressed in the transformants are responsible for the activation, then the addition of α-NF should result in the enhancement of aflatoxin B_1 activation. As shown in Fig. 3, the addition of α-NF to the culture medium resulted in approximately 6-fold enhancement of the cytotoxicity as calculated by IC_{50} values. In contrast, the addition of troleandomycin (TAO), a suicidal substrate of CYP3A enzymes, resulted in decreased cytotoxicity of aflatoxin B_1 (Fig. 4). Based on these results, we confirmed that the CYP3A7 enzyme as well as the CYP3A4 enzyme expressed in CHL cells functioned as an enzyme responsible for the activation of aflatoxin B_1. These cell lines may be useful tools for further studies probing the possible cytotoxicity of other chemicals.

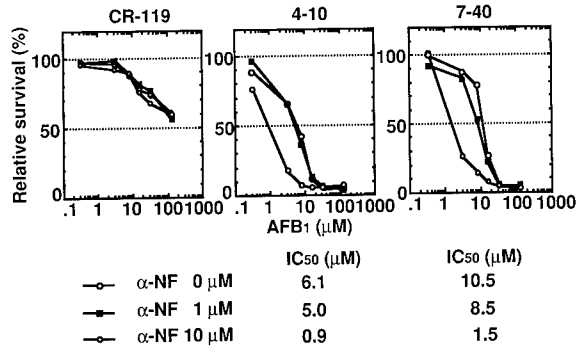


Fig. 3. Enhancement of cytotoxicity of AFB1 by α-NF.

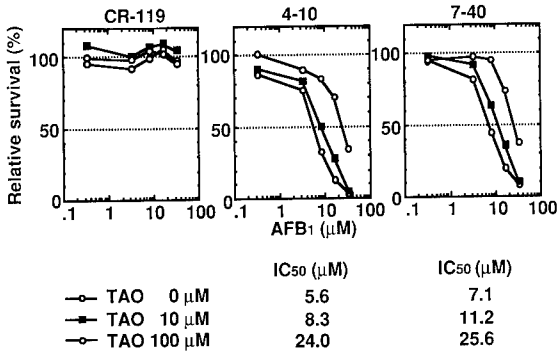


Fig. 4. Decrease of cytotoxicity of AFB1 by TAO.

4. Establishment of cell lines expressing CYP3A7 from hepatocytes derived from CYP3A7-transgenic/p53-knockout mice

Since CYP3A7 is an enzyme present specifically in human fetal livers, it seemed important to test whether transgenic mice carrying the gene demonstrated greater embryo toxicity to known toxicants. For this purpose, we have established strains of transgenic mice by introduction of a plasmid containing *CYP3A7* cDNA downstream of a mouse metallothionein (MT-1) promoter (Fig. 5) (unpublished). CYP3A7 protein was expressed in various organs including the liver, kidney, testis and other organs, depending on the strains. Among the strains, the M-10 line of mice

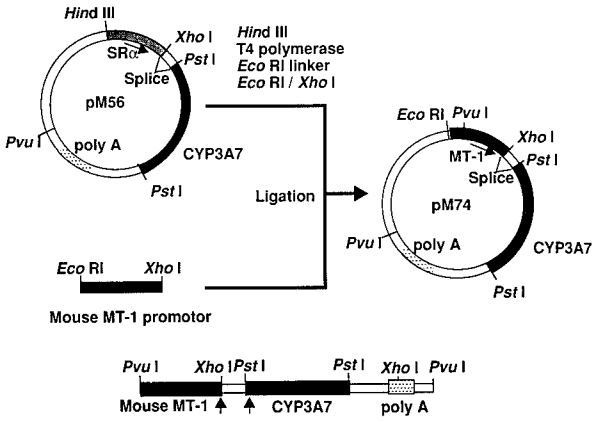


Fig. 5. Construction of a plasmid for transgene introduction in mouse embryos.

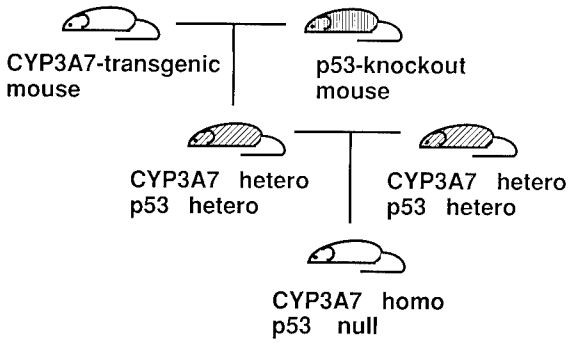


Fig. 6. CYP3A7-transgenic/p53-knockout mouse.

expressed CYP3A7 protein in the liver, kidney and testis.

p53 is a tumor suppressor gene and behaves as a negative growth regulator. Recently, p53-knockout mice were established by gene targeting [7,8]. Fibroblasts from the p53-knockout mice have been established which have acquired immortality [8,9]. In fact, hepatocytes isolated from p53-knockout mice expressed Cyp3a mRNA and proteins for a longer period of time as compared to the cells from wild mice (unpublished).

Based on these results, it was expected that hepatocytes derived from CYP3A7/p53-knockout mice produced by mating of CYP3A7-transgenic mice with p53-knockout mice (Fig. 6) might generate another useful model system for CYP3A7 expression. Thus, the functional capacity of CYP3A7 was examined in the hepatocytes. As shown in Table 1, the activity of CYP3A7 as determined by propoxycoumarin *O*-depropylation was manifested only after culture of the cells in the presence of zinc ion, indicating that the expression was regulated by the MT-1 promoter. These results further support the idea that one can produce immortalized cells expressing desired enzymes by crossing certain transgenic mice with p53-knockout mice. In addition, cell lines expressing multiple enzymes may be possible to engineer by crossing p53-knockout mice with transgenic mice carrying multiple enzymes, themselves produced by mating of other transgenic mouse strains.

Table 1
7-Propoxycoumarin *O*-depropylase activity

Treatment	Activity (pmol/10^6 cells/h)
None	N.D.
$ZnCl_2$ (16 μM)	18.3

The results expressed were the means from 2 independent experiments. Immortalized cells were used for the studies, subsequent to their maintenance for 2 months in culture.
N.D., not detectable (<0.1 pmol).
None, DMSO (0.1%).

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Toxicology Letters

Genetically modified Chinese hamster ovary (CHO) cells for studying the genotoxicity of heterocyclic amines from cooked foods

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Abstract

We have developed metabolically competent Chinese hamster ovary (CHO) cells to evaluate the genotoxicity associated with heterocyclic amines, such as those that are present in cooked foods. Into repair-deficient UV5 cells we introduced cDNAs for expressing cytochrome P450IA2 and acetyltransferases. We then genetically reverted these transformed lines to obtain matched metabolically competent repair-deficient/proficient lines. For a high mutagenic response, we find a requirement for acetyltransferase with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) but not with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). This system allows for both quantifying mutagenesis and analyzing the mutational spectra produced by heterocyclic amines.

Keywords: DNA repair; Mutagenesis; Metabolic activation; Heterocyclic amines; PhIP; IQ

1. Introduction

1.1. Heterocyclic amines in the diet

Diet is generally considered an important component in establishing cancer risk, and one component is the class of heterocyclic amine mutagens, identified in cooked meats, that are potent bacterial mutagens [1,2]. Several of these compounds have been shown to be rodent carcinogens (reviewed in [3]). Among the heterocyclic amines identified in cooked meats (not flame broiled), PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) and MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline) have been detected at the highest levels. PhIP (69 ng/g) and MeIQx (6.4 ng/g) were detected in the greatest quantities in fried fish [4] and in fried steak (48.5 and 8.3 ng/g, respectively) [5]. PhIP was the major heterocyclic amine formed (15 ng/g) in fried hamburgers [6]. In a fried Norwegian meat patty with added creatine, PhIP (62 ng/g) and MeIQx (83 ng/g) also were detected in the highest amounts [7]. Because of the levels of these compounds relative to other heterocyclic amine food mutagens, PhIP and MeIQx may be the most relevant to cancer risk.

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1.2. Metabolism and carcinogenesis

Since the tumorigenic effects of most chemical carcinogens involve metabolic activation processes, cancer susceptibility can be highly dependent upon the makeup of both activation and detoxification enzymes within a given tissue. In humans, interindividual variation in carcinogen metabolism represents a potentially important determinant in risk associated with exposure to carcinogens [8]. For example, variation in the activating potential of human liver microsomes for N-oxidation has been shown for 4,4′-methylene-bis(2-chloroaniline) and 4-aminobiphenyl [9,10] as well as for food-derived mutagens such as IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) and MeIQx [11]. In addition to cytochrome P450 variation, human sulfotransferase levels have been shown to vary in individuals [12], and rates of acetylation in humans vary considerably as well (reviewed in [13]). These variations may be a factor in cancer susceptibility. These findings on variations in activity of xenobiotic-metabolizing enzymes underscore the need for mechanistic studies that could aid in evaluating susceptibility of humans exposed to various carcinogens.

1.3. Heterocyclic amine carcinogenesis and mutagenesis

IQ and MeIQx produced tumors in a variety of tissues including the liver, lung, and intestine [3]. Rats appeared to be more susceptible to the carcinogenic effects of these compounds than mice. In studies with cynomolgus monkeys, IQ produced hepatocellular carcinomas in 100% of the animals under test at the high dose (20 mg/kg) and 70% of the animals at the lower dose (10 mg/kg) [14]. PhIP was shown to produce lymphomas in mice [15] and tumors of the breast and colon in rats [16], but did not produce the class-characteristic liver tumors seen with heterocyclic amines such as MeIQx or IQ, suggesting that the genotoxic or metabolic mechanisms involving PhIP are somewhat different from that of other heterocyclic amines. Moreover, consistent with the in vivo findings, genotoxicity studies using CHO cells supplemented with rat liver S9 for activation indicated that PhIP behaved differently; it was more potent than IQ, MeIQx, or MeIQ (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline) in producing mutations and sister chromatid exchanges even though it was much less mutagenic in the *Salmonella* assay [17]. Recent studies in our laboratory add additional understanding to this initially puzzling result (see Results below). Furthermore, no activity was detected with IQ or MeIQ in *Salmonella* TA98/1,8-DNP$_6$, an acetyltransferase-deficient strain, but PhIP was mutagenic in this strain [18,19].

1.4. PhIP metabolism

Heterocyclic amines are activated to mutagenic forms via N-hydroxylation. Metabolism of PhIP has been characterized in cell-free systems including purified cytochrome P450 and microsomal preparations [20] that converted PhIP to 2 major metabolites, the mutagenic N-hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP) and a C-hydroxylated detoxification product, 2-amino-1-methyl-6-(4′-hydroxy)phenylimidazo-[4,5-*b*]pyridine (4′-hydroxy-PhIP). Formation of mutagenic metabolites from PhIP was isozyme specific; rabbit CYPIA2 (cytochrome P450IA2) and CYPIA1 were shown to convert PhIP to *Salmonella* mutagens [21]. Likewise, rat and rabbit CYPIA1 and CYPIA2 [20,22] were the most active in metabolizing PhIP to *N*-hydroxy-PhIP in vitro. CHO cells that express a mouse CYPIA2 cDNA (see Results) activated PhIP to a genotoxic metabolite(s) [23]. Human CYPIA2 also activated heterocyclic and aromatic amines to mutagenic metabolites [10,24]. In isolated rat hepatocytes, PhIP was metabolized to *N*-hydroxy-PhIP, 4′-hydroxy-PhIP, the sulfate conjugate of 4′-hydroxy-PhIP, and additional unidentified metabolites [18,25]. All direct-acting mutagenic activity in *Salmonella* was attributed to *N*-hydroxy-PhIP. *N*-Hydroxy-PhIP, but not PhIP was genotoxic in CHO cell preparations in the absence of an exogenous metabolizing system [23]. Other data suggest that while N-hydroxylation is important in the genotoxic activity of

PhIP, additional Phase II pathways involved in conjugation appear necessary for adduct formation and mutagenesis [26].

1.5. Derivation of CHO cells deficient in nucleotide excision repair

CHO cells are widely used because of their favorable growth properties and ease of use in mutagenesis studies [17,27]. They have also been valuable for isolating mutant lines that carry specific defects in DNA repair [28,29], especially for the nucleotide excision repair (NER) pathway that acts on UV photoproducts and bulky chemical adducts. The CHO line UV5 was produced by chemical mutagenesis using ethyl methanesulfonate [28] and is defective in the hamster homolog of the *ERCC2* (*XPD*) gene [30], which is present in a single copy in all CHO cells. The particular mutation is a single amino acid substitution (Cys116Tyr), which abolishes the repair function of the protein [31]. Since the ERCC2 protein appears to be an essential protein for transcription by RNA polymerase II and, therefore, cell viability, this mutation must not interfere with the transcription function of the protein. UV5 cells grow well with a doubling time of 13 h compared with 12 h for the wild-type AA8 line [28]. The ERCC2 protein has a DNA helicase activity [32] and is a highly conserved member of the TFIIH transcription factor complex [33]. TFIIH is absolutely required for the incision step of NER, possibly because of its helicase activities (present in ERCC2 as well as ERCC3 [33]) that may unwind the damaged DNA segment before and/or after incision. UV5 cells were the starting line for further genetic modification to produce a sensitive test system.

2. Results and discussion

2.1. Expression of cytochrome P450 in repair deficient/proficient cells

Since bacterial and mammalian mutagenesis studies clearly indicated a requirement for oxidative activation of the heterocyclic amines to mutagenic forms, we modified the UV5 CHO cells by transfecting them with a cDNA expression plasmid carrying the mouse CYPIA2 cDNA, which will provide activation of both PhIP and IQ [34]. Once we had identified a clonal isolate that was efficiently expressing the cDNA, we restored the repair capacity by genetic reversion (see Fig. 1). This approach gave a matched pair of cell lines (UV5P3 and 5P3R2), which should be identical for CYPIA2 expression while differing in repair capacity. Thus, differential sensitivity of these cell lines to killing by a given agent can be attributed to DNA damage and not some other form to toxicity. Using the mouse CYPIA1 cDNA, we also produced a pair of cell lines (UV5P1 and 5P1R1) that were able to activate polycyclic aromatic hydrocarbons [35].

2.2. Sensitivity of P450-expressing cells to killing and mutagenesis by PhIP and IQ

Fig. 2 summarizes the sensitivity of our cell lines to killing by PhIP and IQ. We have taken as a measure of effective dose the D_{37} value, which is the dose that reduced the survival to 37% during a 4-h exposure in the presence of rat liver S9 or during a 48-h exposure in the case of P450-expressing cells. In the absence of S9 frac-

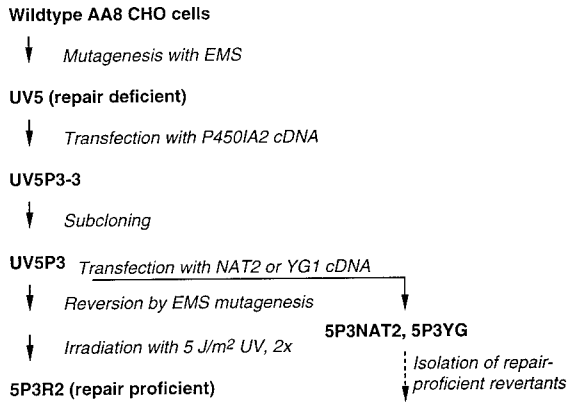


Fig. 1. Derivation of the cell lines used in this study. AA8 CHO cells are a clone that is heterozygous at the *aprt* locus that can be used for measuring mutations to 8-azaadenine [27]. The derivation of UV5P3 and 5P3R2 was described earlier [34], and the derivation of 5P3NAT2 and 5P3YG was recently performed using UV5P3 as the starting material.

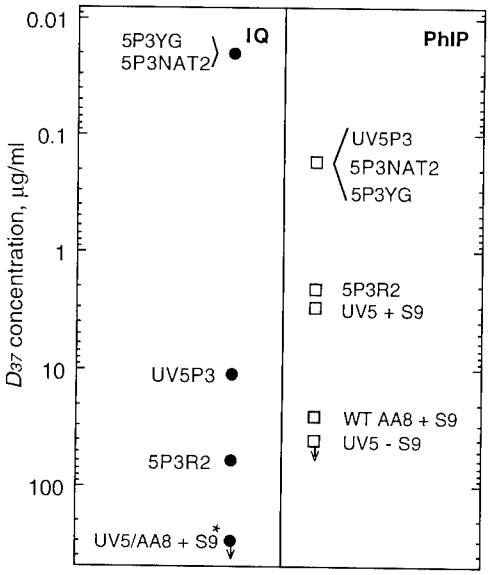


Fig. 2. Relative sensitivity to PhIP and IQ of the CHO cell lines indicated in Fig. 1. Sensitivity is measured as the D_{37} dose, i.e. the dose required to reduce the colony forming ability to 37% relative to untreated controls. The sensitivity of the various cell lines to PhIP is shown in the upper part of the figure and the sensitivity to IQ in the lower. In the case of UV5 and AA8 cells exposed to IQ in the presence of S9 (*), the killing showed a plateau at ~50% for doses up to the limit of solubility; therefore, there was no D_{37} dose. With one exception (AA8 cells in the presence of S9 fraction) cell killing was always accompanied by significant mutation induction. Therefore, doses that produced clear toxicity also produced significant mutagenesis.

tion, UV5 cells showed no toxicity up to the solubility limit for IQ (see Fig. 2 legend). For PhIP, we previously reported that UV5 cells were clearly susceptible to killing and mutation induction at the *hprt* locus mediated by rat liver S9 fraction [17]. However, UV5P3 cells were >10-fold more sensitive than UV5 in the presence of S9 and were ~15-fold more sensitive than the repair-proficient 5P3R2 cells [17,34]. We detected mutation induction in UV5P3 cells at PhIP doses as low as 0.1 µg/ml. 5P3R2 cells showed significant but weak mutagenesis at doses above 2 µg/ml. In the presence of S9 fraction, weak mutagenesis was also reported in repair-proficient CHO cells at the *dhfr* locus and in human TK6 cells at the *tk* and *hprt* loci [36,37]. The cytotoxicity dose dependence of UV5 and UV5P3 cells exposed to *N*-hydroxy-PhIP was identical, indicating that N-hydroxylation was the initial activation pathway involved in PhIP genotoxicity. Microsomal preparations from UV5P3 cells, but not UV5 cells, were specifically shown to convert PhIP to *N*-hydroxy-PhIP [23].

Initially we found that IQ, as an extremely potent mutagen in the Ames/*Salmonella* test, was only slightly toxic to UV5 and AA8 cells in the presence of S9 fraction. A dose dependence on repair was seen for mutagenesis but not killing [38], and induced mutations were readily apparent only in UV5 cells. P450-expressing UV5 cells showed greater sensitivity (see Fig. 2) [34], but still the magnitude of mutation induction was low in both UV5P3 and 5P3R2 cells, suggesting that IQ was not activated to the same mutagen (i.e. intermediate) as in *Salmonella*.

2.3. Expression of acetyltransferases relevant to IQ and MeIQx metabolism

Recently, it was found that the expression of *N*-acetyltransferases and *O*-acetyltransferases in CHL hamster cells rendered them more sensitive to killing by IQ in the presence of rat liver microsomal fraction [39]. *Salmonella O*-acetyltransferase was more effective than the human *N*-acetyltransferases (NAT1 and NAT2), inducing toxicity and micronuclei in the range of 1–4 µg/ml IQ. To test the idea that the important missing activity in our CHO system was acetyltransferase, we obtained plasmids used by Watanabe (kindly provided by Drs. T. Nohmi and T. Deguchi), each of which expresses acetyltransferase. Two of these plasmids were introduced into UV5P3 cells as outlined in Fig. 1. The resulting cell lines, 5P3YG and 5P3NAT2 (with bacterial and human transferases, respectively), proved to be extremely responsive to IQ, showing D_{37} doses of ~0.02 µg/ml. This sensitivity represented ~1000-fold increase compared with UV5P3 cells! There was no significant difference in sensitivity between the lines 5P3YG and 5P3NAT2. In parallel with the toxicity, we observed a similar shift in sensitivity to mutagenesis and high levels of mutation induction (R. Wu. and J. Felton, unpublished results), suggesting that DNA adducts were being formed very

efficiently in the cells. Neither the 5P3YG or 5P3NAT2 cell line was more sensitive to PhIP than the parental UV5P3 line, indicating the lack of requirement of acetyltransferase activity in the activation of PhIP. This result was expected based on the bacterial data from the *Salmonella* strains differentially expressing *O*-acetyltransferase. In summary, the behavior of the transferase-expressing lines with PhIP and IQ was qualitatively similar to that of the Ames/*Salmonella* reversion assay, in which IQ is ~100-fold more potent than PhIP. 5P3NAT2 cells showed about a 10-fold difference in potency for the 2 compounds.

2.4. Mutational spectrum with PhIP in UV5P3 cells

The cell lines we have used are well suited to detailed mutagenesis studies because mutations can be analyzed at both the *aprt* and *hprt* loci. The small *aprt* gene is especially convenient, and we devised a method to sequence it directly from polymerase chain reaction products [40]. In an initial study we examined the spectrum of *aprt* mutations induced by PhIP in UV5P3 cells. We found a very surprising result in that most of the mutations (75%) occurred in only 3 sites. All the mutations were single-base transversions; 2 of the 3 were C:G to A:T changes. This pattern is reasonable based on the propensity for heterocyclic amines to bind guanine. During replication, the adducted G can pair with A instead of C according to the A rule for insertion at non-instructional sites [41]. All 3 of these hotspot mutations result in stop codons or charge changes in the protein. None of the spontaneous mutants had alterations at these sites [40]. The PhIP-induced mutations in these repair-deficient CHO cells were unique and specific, and suggest that these base sequences may be targets in other genes critical for carcinogenesis.

3. Future directions

We plan to further develop the CHO system in several respects. (1) We plan to isolate repair-proficient lines derived from cells that express both the CYPIA2 and acetyltransferase genes. We can then compare the efficiency of repair of IQ or MeIQx adducts with the repair of PhIP adducts. We will attempt to relate any significant differences in repair to differences in mutagenesis and carcinogenesis. (2) For analysis of mutational mechanisms, we plan to examine the mutational spectrum produced by PhIP in repair proficient cells. Preliminary results suggest that there are major differences and that the hotspots are not detected, which suggests that repair is extremely important in determining the final spectrum of mutagenic adducts in normal cells. (3) Studies are needed on MeIQx, which is more relevant in terms of human exposure in the Western diet, to see if it behaves similarly to IQ in terms of adduct repair and mutagenic potency.

Acknowledgements

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Injury and repair as opposing forces in risk assessment

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Abstract

Recent advances in our understanding of the toxicodynamic events that follow infliction of injury have helped us to bridge the link between the tissue injury and the final outcome of that injury. In addition to infliction of tissue injury, toxic chemicals induce a biological compensatory response of tissue repair intended to overcome tissue injury through healing. Since stimulation of tissue repair is a simultaneous response accompanying injury, measuring this response in addition to quantifying injury might be helpful in tomorrow's risk assessment. Studies with model hepatotoxicants such as thioacetamide and CCl_4, where tissue repair as well as injury were measured, reveal that endogenous mechanisms that drive the tissue repair response are responsible for more than just compensation for tissue injury. Up to a threshold dose, tissue repair is stimulated in a dose-dependent manner, and above this threshold it is both delayed and diminished. During this delay, tissue injury progresses unabated leading to tissue destruction and animal death. While dose-related stimulation of tissue repair leads to recovery, delayed and diminished tissue repair seen at the high doses leads to tissue destruction and animal death. These findings impact on the currently used maximum tolerated doses (MTDs) in cancer bioassays. MTDs represent maximal stimulation of cell proliferation thereby enhancing the likelihood of errors in DNA replication. Measuring tissue repair and injury as simultaneous biological responses to toxic agents might increase the usefulness of dose-response paradigms in risk assessment.

Keywords: Tissue repair; Chlordecone; Carbon tetrachloride; Dose-response for tissue repair; Thioacetamide; Maximum tolerated doses; Risk assessment

1. Introduction

A major issue in risk assessment is the mechanistic basis for quantitative assessment of risk from exposure to single or combinations of chemicals. Of particular interest in public health is the risk from exposure to chemicals at environmentally relevant levels. While we have made enormous progress during the last few decades in our understanding of the mechanisms by which toxic chemicals initiate injury, our understanding of the toxicodynamic mechanisms that occur in the tissues after the infliction of injury has lagged considerably.

Work with 2 chemicals as the simplest examples of mixture toxicology has enabled scientists to uncover mechanistic aspects of the toxicology of single as well as mixtures of chemicals. One such model, where prior dietary exposure to a nontoxic level (10 ppm) of the pesticide chlordecone (Kepone®) results in a 67-fold amplification of CCl_4 lethality in rats has been well studied [1–3]. Examples of toxic interactions where the interactive toxicity does not

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Table 1
Mechanism of chemical interactive hepatotoxicity in relation to its consequence to animal survival/death

	Interaction	Mechanism of infliction of liver injury	Effect on tissue repair	Consequence to animal survival/death
(1)	Chlordecone-potentiated CCl_4 toxicity	CCl_4 bioactivation is slightly increased	Tissue repair is suppressed	Animals do not survive
(2)	Phenobarbital-potentiated CCl_4 toxicity	CCl_4 bioactivation is considerably increased	Tissue repair is increased	Animals survive
(3)	Isopropanol-potentiated CCl_4 toxicity	CCl_4 bioactivation is considerably increased	Tissue repair is increased	Animals survive
(4)	Dibenamine and CCl_4	CCl_4 bioactivation is slightly decreased	Tissue repair is increased	Animals survive

have a consequence on animal survival are also known (Table 1). For example, prior exposure to phenobarbital and subsequent exposure to very low levels of CCl_4, chloroform or bromotrichloromethane results in highly increased liver injury [4]. However, this highly exaggerated liver injury does not lead to increased lethality in exposed animals. Likewise, prior exposure to alcohols is known to result in highly increased liver injury of halomethane compounds [5,6]. Of mechanistic interest here is the observation that exposure to a homologous series of alcohols up to 3 carbon length leads to increased liver injury, without leading to increased animal lethality [7]. Therefore, it appears that this class of interactive toxicity and the mechanisms underlying these interactions are different in comparison to the first class of interactions described above. The mechanisms responsible for animal survival in spite of highly exaggerated liver injury are only now becoming known [8,9]. A third type of interaction is represented by prior exposure to dibenamine which results in protection against CCl_4 hepatotoxicity and lethality [10]. Although all of the information is not available, it appears that in addition to decreased bioactivation of CCl_4 that results in decreased infliction of injury, in this model increased tissue repair also seems to play a role (Table 1) in rescuing the animals from death.

2. Examples of toxicity models where tissue repair is of critical importance

The mechanism underlying the chlordecone-amplified toxicity of halomethanes was extensively investigated during the 1980s [1,12–14]. Close structural analogs of chlordecone such as mirex and photomirex do not have the propensity of chlordecone in this regard. While phenobarbital and halomethane combination results in highly increased liver injury, even more than that observed with chlordecone and halomethanes, this injury is of no consequence to animal survival. Also, after extensive investigation, it became apparent that neither enhanced bioactivation of CCl_4, increased lipid peroxidation nor a combination of these 2 mechanisms and a variety of other considerations related to the initial event of infliction of injury, can explain the highly amplified nature of the chlordecone + halomethane interactive toxicity [1,4].

Additional studies led to the discovery that ordinarily a low dose of halomethane such as CCl_4 is nonlethal because of stimulated tissue repair that occurs simultaneously when the initial events inflict liver injury. It was found that maximal liver injury occurs at a different time when CCl_4 is administered at low doses in comparison to the maximal time of injury when much higher doses are administered [1]. Subsequent studies have revealed that the recovery from injury inflicted by a low dose of CCl_4, chloroform, or bromotrichloromethane is due to the stimulation of cell division that occurs in 2 phases [1,4]. First, an early burst of cell division occurs as early as 6 h after CCl_4 administration with a second, larger wave of cell division occurring 36–48 h after administration of CCl_4. It is clear that the early burst of cell division is due to the mobilization of a small number of hepatocytes which are present normally in the liver in G_2 phase [14,15]. If the animals were exposed to

food contaminated with 10 ppm chlordecone for 15 days prior to the administration of a single low dose of CCl_4 (100 μl/kg, i.p.) the early phase of cell division was abolished. It is known that during cell division a number of growth factors and proto-oncogenes are overexpressed and these products of gene expression profoundly influence by facilitating division of other cells in the tissue [16–19]. Therefore, if the early phase of cell division does not occur the mechanism and signals necessary to 'prime' the neighboring cells would be unavailable. This results in progression of injury due to lack of a biological mechanism to restrain injury [20]. By 24–36 h while the second phase of cell division does occur, in the face of the unrestrained progression of injury, this tissue repair becomes far too little and too late to restrain the accelerated progression of injury. It is now known that the reason for failed early phase cell division in animals receiving the combination of chlordecone and CCl_4 is the lack of cellular energy [21]. At 6 h after CCl_4 administration the available ATP is precipitously decreased in the livers of rats receiving chlordecone and CCl_4 combination thereby incapacitating the cells from dividing. Based on experiments in which the external sources of energy were provided, availability of cellular energy results in restoration of the early phase cell division, and the animals survive [1,21].

2.1. Toxicological interactions leading to increased liver injury but not increased death

Exposure to higher levels of phenobarbital but the same low level of CCl_4 results in almost twice as much liver injury, but this does not lead to increased animal mortality [22]. Depletion of ATP does not occur in these livers [23,24]. Therefore, the only consequence of this highly toxic liver injury is to postpone the early phase cell division until 24 h. However, the second phase of cell division is greatly stimulated [24]. In combination, this wave of highly stimulated cell division and tissue repair leads to systematic restoration of hepatolobular structure, and function, followed by full animal recovery. Studies with methanol, ethanol and isopropanol-potentiated CCl_4 liver injury have revealed that while liver injury of CCl_4 is increased, this does not lead to higher mortality [7,25]. Recent studies with isopropanol-potentiated CCl_4 liver injury reveal the reason behind this. While exposure to isopropanol results in increased bioactivation of CCl_4 that expectedly leads to higher infliction of higher liver injury, simultaneously, a highly stimulated tissue repair works to restrain liver injury on the one hand and to restore hepatolobular structure and function on the other, leading to recovery from injury and animal survival [8,9]. While many toxic chemicals are known to stimulate cell division and tissue repair [26], the above 2 examples of chemical interactions illustrate the importance of stimulated tissue repair and the dynamic interaction between the 2 opposing forces that determine the ultimate outcome of toxic injury (Table 1).

Regardless of the mechanisms that lead to infliction of tissue injury, the biological events that occur beyond that step determine whether the progression or regression of that injury will occur, which in turn determines the ultimate outcome. Thus, if tissue repair is permitted to occur, injury is restrained, tissue structure and function are restored and animal recovery occurs. On the other hand, if tissue repair is blocked, unrestrained progression of injury leads to further deterioration of the tissue and ability to survive. While the above 2 examples depict 2 ends of the spectrum of the role played by tissue repair, other examples that fall in between this spectrum might also emerge. For example, recent studies with dibenamine + CCl_4 interaction suggest (Table 1) that both decreased bioactivation as well as increased tissue repair might play a role in rescuing the animals from CCl_4-induced lethality [27].

2.2. Stimulation of tissue repair is a function of animal species/strains

What do we know about the ability of chemicals to induce cell division and tissue repair? While many chemicals are known to induce tissue repair [26], they differ in their ability to stimulate cell division and tissue repair with regard to the magnitude of the response. The

time at which the cell division is stimulated may also be a function of the chemical characteristics, although this is also known to be dependent on the animal characteristics such as animal species [28,29] and age [30,31]. Species and strain differences in the extent to which tissue repair is stimulated after exposure to toxicants are thought to play a significant role in the species and strain sensitivity to toxicants [10,28,32,33].

3. Tissue repair is the key to animal survival in autoprotection and heteroprotection models

If stimulated tissue repair is critical for animal survival upon administration of a toxic chemical at a lethal dose, one should be able to protect animals from the lethal action of an ordinarily lethal dose by pre-placing tissue repair induced by a low dose of the same compound. Recent studies have revealed that CCl_4 autoprotection is due to the stimulation of cell division and tissue repair induced by a priming low dose of CCl_4 administered 24 h prior to the administration of the lethal challenge dose [1,34,35]. Antimitotic intervention with the stimulation of tissue repair by the priming dose with colchicine leads to abolition of autoprotection [36,37]. In these experiments, neither bioactivation nor the metabolism and disposition of CCl_4 were affected [20]. Exposure to phenobarbital is known to postpone the early phase of cell division to 24 h [22]. If stimulated cell division is the primary mechanism responsible for CCl_4 autoprotection, administration of the priming dose in phenobarbital-induced animals should lead to a postponement of maximal autoprotection by approximately 24 h. Autoprotection experiments with phenobarbital-induced animal models revealed that maximal autoprotection was delayed by 24 h in comparison to autoprotection in naive animals [34]. These experiments also suggested that destruction of cytochrome P450 by the priming dose of CCl_4 was not the primary mechanism of autoprotection. The discovery of stimulated tissue repair as the underlying mechanism of CCl_4 autoprotection has far-reaching implications in toxicology of chemical combinations and chemical mixtures. It was of interest to investigate if the same mechanism could explain autoprotection of other structurally and mechanistically dissimilar chemicals. The mechanism for thioacetamide autoprotection is also stimulated cell division and tissue repair by the priming dose of thioacetamide [38]. The mechanism of 2-butoxyethanol autoprotection is due to increased appearance of newly formed red blood cells in the animal models [39]. Therefore, work with structurally diverse model toxicants known to inflict tissue injury by equally diverse mechanisms points to the critical importance of toxicant-stimulated tissue repair in recovery from even massive injury [1].

3.1. Mechanism of heteroprotection

The above experiments suggested that it should be possible to protect animals from a lethal dose of a compound by pre-placing stimulated tissue repair using a small dose of any toxic chemical. Studies in which a low dose of thioacetamide was employed to pre-place stimulation of cell division have confirmed this possibility. Rats receiving a lethal dose of acetaminophen 36 h after administration of thioacetamide (50 mg/kg) are fully protected from acetaminophen-induced lethality [40]. Detailed studies revealed that neither the disposition of acetaminophen nor the bioactivation and infliction of acetaminophen liver injury are affected by prior treatment with thioacetamide. Pre-placed stimulated tissue repair appears to sustain continued tissue repair, restrain acetaminophen-induced liver injury and enable the animals to recover from normally lethal doses of acetaminophen [40].

These findings have been pivotal in gaining new insights into biological events that appear to follow infliction of tissue injury. While the mechanisms that inflict injury have represented most significant advances in toxicology, these new findings are likely to help toxicologists to break new ground.

4. Toxicant-inflicted injury and stimulated tissue repair are opposing toxicodynamic forces in predictive toxicology

The above examples suggest that injury and

tissue repair are simultaneous but opposing parallel responses to administration of toxic chemicals. A dose-response relationship that encompasses the characteristics of exposure and the spectrum of effects in a correlative manner is the most fundamental concept of toxicology. This concept is used in predictive toxicology and consequently is a very basic principle used in risk assessment. If stimulated cell division and tissue repair are critical in predicting the ultimate outcome of toxic injury, then in addition to measuring injury in response to increasing doses of chemicals, it would also be advantageous to measure the simultaneous but opposing response of stimulated tissue repair. This shifting dose-response paradigm would be more precise and accurate in predicting the final outcome of toxic injury. However, if in developing dose-response relationships for toxic chemicals, we measure only toxic injury against a series of increasing doses, this information may be incomplete and may lead to erroneous predictions. To test the hypothesis that measuring both important and critical players (injury and tissue repair) that seem to dynamically interact in an opposing manner would yield a more valuable dose-response paradigm, we conducted some studies with model hepatotoxicants [41,42].

4.1. Dose-response studies with thioacetamide

For dose-response studies with thioacetamide, a 12-fold dose range was selected (50, 150, 300 and 600 mg/kg, i.p.). The first 3 doses of thioacetamide were known to cause centrilobular hepatotoxic injury, but if left alone these animals survive. Rats injected with the highest dose (600 mg/kg, i.p.) fail to survive. This allows sufficient spread of time-course to carefully study the progression of each of the 2 critical events. These studies revealed that after administration of a 6-fold dose range (50, 150, and 300 mg/kg), maximal liver injury was evident at 24 h after injection of thioacetamide. Most interestingly, the injury associated with all 3 doses was identical at 12 and 24 h. In other words, a dose-related difference in liver injury as assessed by elevation of alanine aminotransferase (ALT) or sorbitol dehydrogenase (SDH) was not evident. After experiencing sustained liver injury during the ensuing 24 h, the animals recovered from this injury by 72 h. Histopathological examination of liver sections revealed peak hepatocellular necrosis around 24 h and by 72–96 h there was no evidence of liver injury. All of these animals go on to survive the challenge of thioacetamide. Rats receiving the highest dose (600 mg/kg) experienced much less liver injury at 12 h in comparison to the 3 lower doses, reached comparable injury as the other 3 doses by 24 h, and a more or less sustained level of injury over the next 24 h. It was not until after 48 h that this group of rats experienced a dramatic increase in hepatotoxic injury. This injury progressed through 96 h and thereafter within the next 3 days all of these animals experienced mortality [41].

Stimulation of tissue repair associated with administration of thioacetamide was measured by [^{3}H]thymidine incorporation in hepatocellular nuclear DNA over the same time-course employed for assessment of liver injury. Stimulation of cell division and cell cycle progression were measured also by immunohistochemical analysis of proliferating cell nuclear antigen (PCNA). With the 6-fold dose range (50, 150, and 300 mg/kg), with each increment in dose, there was an incremental stimulation of S phase synthesis as assessed by [^{3}H]thymidine incorporation. While the lowest dose (50 mg/kg) stimulated S phase synthesis with a peak at 36 h after administration, a 3-fold higher dose resulted in higher stimulation at 36 h and sustained at 48 h. The 300 mg/kg dose of thioacetamide yielded a higher peak stimulation of S phase synthesis but not until 48 h. While there was an increment in the stimulation of tissue repair with each higher dose, as the dose increased, there appeared to be a delay in the time at which the maximal stimulation of tissue repair occurred [41]. While there was no significant difference between the 3 doses in liver injury, stimulated tissue repair differed markedly among the 3 doses. Rats receiving the highest dose of thioacetamide (600 mg/kg) did not show any stimulation of tissue repair until 72 h. And then this response was greatly attenuated. Therefore, it appeared that very high doses of thioacetamide resulted in much delayed and

greatly diminished stimulation of tissue repair. It should be recalled that the injury greatly accelerates in rats receiving this dose at about 48 h after its administration. This suggests that in the absence of stimulation of tissue repair, unrestrained progression of injury occurs indicating that tissue repair stimulation is too little and too late to rescue these animals from progression of liver injury. Detailed analysis of liver sections by PCNA immunohistochemical studies revealed that the lower doses result in very prompt cell cycle progression leading to cell mitosis while the highest dose does not. A fascinating observation of this study is the dynamic relationship between the tissue repair response and the progression of injury. It is only after failure to elicit a prompt tissue repair response that an accelerated progression of liver injury becomes evident culminating in liver failure and death. Tissue repair due to 6-fold dose-range peaked between 24 and 48 h, but with 600 mg/kg it was much delayed and significantly decreased. Thus, a failure in timely and adequate appearance of tissue repair leads to an unrestrained progression of injury in the animals receiving a dose beyond the threshold for stimulation of tissue repair [41].

4.2. Dose-response studies with carbon tetrachloride

Similar studies were conducted with a 40-fold dose range of CCl_4 [42]. The dose ranges used for the CCl_4 study were 0.1, 1, 2, 3, and 4 ml/kg (i.p.). The findings of this study were also similar to those of the thioacetamide study. Rats receiving 4 ml CCl_4/kg experienced 80% mortality within 4 days. It was routinely observed that 20% of the animals consistently survived this challenge dose. The mechanism that enables these animals to overcome even massive injury appears to be an extraordinarily high stimulation of cell division as determined by [^{3}H]thymidine incorporation and also by PCNA studies [42]. Therefore, for the 2 structurally and mechanistically dissimilar chemicals, it is possible to say that including a measure of tissue repair along with the measure of injury allows the dose-response paradigm to predict quite accurately the final outcome of toxicant-induced injury.

4.3. Dose-response relationships for injury and tissue repair

In the classic dose-response paradigm, if we now also introduce the measure of the tissue repair stimulation as the biological event opposing injury, we obtain 2 sets of dose-response curves (Fig. 1). At lower doses, as injury begins, there is a simultaneous but opposing tissue repair response allowing the animals to overcome that injury. Predictably, these animals suffer from injury but are rescued from progressive injury and death. With higher doses, one reaches a threshold where any additional increment in the dose results in 2 adverse effects. First, the stimulation of tissue repair, which is delayed with each incremental dose, is now much too delayed. Second, the amplitude of the tissue repair response is diminished. Therefore, decreased stimulation of tissue repair results in unrestrained progression of injury and animal death.

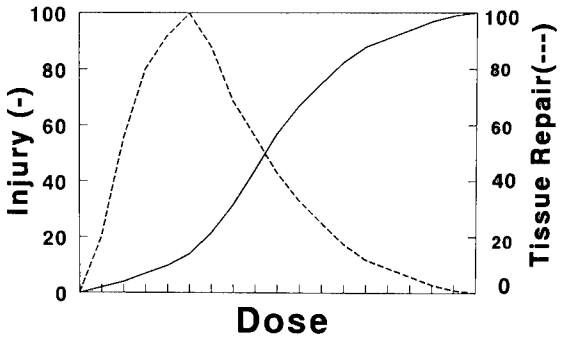


Fig. 1. A typified dose-response relationship between the 2 opposing forces of inflicted injury and stimulated tissue repair upon exposure to a toxic chemical. As the dose increases tissue repair is increased allowing recovery from tissue injury. When the dose exceeds the threshold, tissue repair is attenuated and delayed, allowing injury to progress in an unrestrained manner, leading to organ or tissue failure and animal death. Quantifying both injury or adverse effect as well as stimulated tissue repair simultaneously as a dose-response relationship might be helpful in assessing the outcome of the interaction between these 2 opposing forces. The dose-response relationship can be used to explain inter-individual differences, just as a population response.

In addition to the use of the dose-response curve for prediction of the ultimate outcome in individual subjects, such a response can also be used for prediction in a population.

5. Factors that affect tissue repair

A number of factors are known to affect the extent to which tissue repair is stimulated upon toxicant exposure. Among these factors there are those that can be associated with the chemical characteristics of the toxicant. Then there are those that are associated with the host animal exposed to these toxic chemicals. In addition to the structural features of the toxic chemical, the mechanistic features of the toxicant might also be involved. Although not much is known in this area, it is known that in general regardless of the mechanism by which chemicals afflict injury, stimulation of cell division and tissue repair occur. Among the chemicals that are known to stimulate tissue repair are galactosamine, CCl_4, chloroform, heavy metals, bromotrichloromethane, acetaminophen, ethylene dichloride, thioacetamide, dimethylnitrosamine, and a variety of other chemicals belonging to an equally diverse group of toxic chemicals [26]. The differences between these chemicals might be the time at which tissue repair is stimulated after the episode of exposure. It is not known whether the stimulation of tissue repair occurs primarily as a result of the action of parent compound, or primarily as a result of the action of toxic metabolites, or a combination of both. This area needs further investigation. It would be logical to assume that in addition to the parent compounds the toxic metabolites also have the capability to induce tissue repair. Dose of a chemical is also known to affect the tissue repair response. Early studies revealed that the reason why a low dose of a toxic chemical is not 'lethal', meaning it has no consequence on animal survival, is because of the exacting level of tissue repair stimulation which enables the animals to recover from that injury [1–3]. Subsequent studies in which tissue repair response was investigated upon exposure to higher doses of chemicals revealed that at higher doses the chemicals inhibit tissue repair [41–43]. It follows that the reasons for the lethal action of higher doses are the delay and diminution of the tissue repair response that otherwise seems to promptly accompany the infliction of injury [1,41,42]. Diminished tissue repair response appears to lay the ground for unrestrained progression of injury, thereby leading to failure of the animals to survive (Fig. 1).

6. Conclusions

In conclusion, work with a number of experimental hepatotoxicants indicates that exposure to toxic chemicals leads to 2 responses: first, toxicant-inflicted tissue injury occurs; second, a simultaneous but opposing tissue repair response reponsible for recovery from injury is known to be stimulated, inhibited or unaltered upon exposure to individual or mixtures of chemicals. When tissue repair is inhibited, even an inconsequential level of tissue injury may lead to fulminant liver failure and animal death from even a nonlethal exposure to a hepatotoxicant. When tissue repair is unperturbed, the outcome may be mainly dependent on bioactivation and other mechanisms responsible for infliction of injury. When tissue repair is augmented, tissue injury becomes inconsequential to animal survival even though much higher tissue injury might be inflicted. Studies with interactive models of toxicity using binary mixtures of chemicals reveal that examples of each of the above sequel can be found. Work with other models of chemical interactions such as autoprotection and heteroprotection underscores the importance of stimulated tissue repair in the outcome of toxic injury. These findings suggest that animal survival is possible due to augmented tissue repair by a priming low dose of a toxicant. Additional studies indicate that 2 distinct types of benefit are derived from stimulated tissue repair. First, new cells are available for restoration of tissue structure and function by replacing the dead or dying cells. Second, the new cells are resilient to the action of toxicants and this may play a role in restraining injury.

Because toxicant-induced injury and stimulated tissue repair are simultaneous but opposing responses, measuring both of these responses in a dose-response paradigm is likely to increase the precision of predictive toxicology. Work with structurally and mechanistically diverse chemical toxicants suggests that such dose-response paradigms might be useful in risk assessment. Factors such as exposure dose, macronutrient status and age are known to influence the tissue repair status and thereby affect the ultimate outcome of toxicity. Such quantitative relationships are likely to be helpful in accounting for inter-individual variability in toxicity. Furthermore, strain/species differences in sensitivity to toxicants might be explained by strain/species differences in the tissue repair response elicited after exposure to toxicants. Therefore, in addition to the well-known strain/species differences in the mechanisms of infliction of injury, the quantifiable differences in tissue repair might allow a more precise inter-species extrapolation of data on a more complete scientific basis. Current risk assessment which does not take into account the toxicodynamics of tissue repair will be more precise by incorporating this in tomorrow's risk assessment.

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Biologically based dose response model for hepatic toxicity: a mechanistically based replacement for traditional estimates of noncancer risk

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Abstract

Uncertainty in risk assessment can be reduced by increasing the use of relevant data specific to the particular xenobiotic and exposed organism. We describe the development of a preliminary, mechanism-based exposure response model for chloroform hepatotoxicity consisting of toxicokinetic (TK) and toxicodynamic (TD) submodels. The TK submodel is based on an existing physiologically based toxicokinetic (PBTK) model for chloroform. The TD submodel consists of an empirical function linking tissue dose, defined by the PBTK model, with hepatocyte killing and subsequent regenerative cellular replication. Chloroform-induced cell killing was inferred quantitatively from dose-response hepatic labelling index studies conducted in female B6C3F1 mice and male F344 rats. The overall model was scaled to humans by conventional scaling of the TK submodel and by using the TD submodel as is, i.e. as developed from the rodent data. The resulting human model was used to analyze a case of human poisoning which developed after repeated ingestion of large doses of cough syrup containing chloroform and alcohol. The model predicted the observed toxic response after the capacity for chloroform metabolism was increased by a factor of 3 from the value estimated using human liver microsomes. This is an acceptable adjustment of this parameter, given the uncertainty associated with the extrapolation from microsomes and the coexposure to alcohol. This preliminary result is encouraging, suggesting that the model, at its current stage of development, is able to approximate actual human risks of hepatotoxicity from chloroform exposure. The extensive use of data on chloroform TK and cytolethality-induced regenerative cellular replication for model development suggests that the model has reduced uncertainty relative to the current U.S. EPA oral reference dose (RfD) calculation for chloroform, which does not use any mechanistic or dose-response data.

Keywords: Chloroform; Hepatotoxicity; Quantitative; Mechanism-based model; Noncancer risk assessment; Reduced uncertainty

1. Introduction

For a given exposure scenario, the risk of an adverse health effect is determined by the chemistry of the xenobiotic and the biology of the exposed organism. Default approaches to risk assessment typically utilize little such information and are correspondingly highly uncertain. This uncertainty can be reduced by increas-

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ing the use in the risk assessment model of relevant data specific to the particular xenobiotic and exposed organism. In this manuscript, we describe the use of this strategy for the development of a preliminary, mechanism-based exposure response model for chloroform cytotoxicity.

2. Generic issues in the development of a risk assessment model

Any model of the overall exposure-response relationship can be conveniently divided into 2 submodels, one for the relationship between exposure and dose to target tissue (toxicokinetics; TK) and one for the relationship between target tissue dose and the response of interest (toxicodynamics; TD). This division is useful in several ways. Some phenomena, such as route-to-route differences in systemic toxicity, are entirely a function of TK, and the a priori distinction between TK and TD serves in such cases to focus attention on the appropriate aspect of the overall exposure-response relationship. In addition, characterization of TK is required before the role of TD in, for example, interspecies differences in toxicity can be understood. Any analysis of TD behavior is potentially confounded by TK unless the role of the latter has been adequately characterized.

In general, more tends to be known about TK than TD for most chemicals. Mechanism-based models are thus likely to focus on TK and contain relatively less information about the subsequent TD events. Strategies for scale-up of a risk assessment model from laboratory animals to humans can take advantage of this difference in level of understanding. For example, a mechanism-based TK submodel may be sufficiently well characterized that its scale-up can be based on relevant human data collected in vivo or in vitro. Conversely, a relatively less well-characterized TD submodel is likely to limit the options available for its scale-up. For cases such as this, the TK-TD distinction allows different scale-up strategies for the TK and TD submodels. This approach is taken in the chloroform model described below.

3. Development of a mechanism-based risk assessment model for chloroform

The first goal of the model development process described here was to obtain a quantitative, mechanism-based model that accurately predicts the hepatotoxicity of chloroform in exposed mice and rats. Once this rodent model was developed, it was scaled to the human case, and a limited test of its ability to predict human toxicity was conducted.

3.1. TK submodel

Corley et al. [1] described a physiologically based toxicokinetic (PBTK) model for chloroform that draws on quantitative, physiological, and chemical-specific information to depict the processes controlling the dose of chloroform to liver and kidney, important target organs for the acute toxic effects of this chemical. The PBTK model can be thought of as describing the mechanism, at a particular level of biological detail, that determines the exposure-tissue dose relationship for chloroform. For consistency, the PBTK model is hereafter referred to as the TK submodel.

3.2. TD submodel

The TD submodel for chloroform hepatotoxicity consists of an empirical function linking target tissue dose with the response of interest, in this case regenerative cellular replication. We first consider the definition of target tissue dose and then the data on chloroform-induced regenerative replication.

3.3. Target tissue dose

The main metabolites of chloroform are phosgene and HCl, both of which have very short biological half-lives [2]. Phosgene should form primarily labile macromolecular adducts, and any contribution by the HCl metabolite to chloroform toxicity would presumably be due to transient, localized shifts in intracellular pH. Thus the major metabolites and macromolecular adducts resulting from chloroform exposure are expected to be transient. The measure of dose in the target tissue should be consistent with these

characteristics of the metabolites, which are the presumed ultimate toxicants generated upon exposure to chloroform. In a previous cytolethality model for chloroform, Reitz et al. [3] found that a measure of tissue dose that reflected rate of metabolism rather than, for example, total amount metabolized, was the better predictor of both quantitative histopathology and chloroform-stimulated regenerative cellular replication. The present model defines the target tissue dose surrogate as the amount of chloroform metabolized over the interval ($t - 30$ min, t), where t is the current time. This dose measure is similar to that used by Reitz et al. in that it correlates better with rate of metabolism than with total amount metabolized, at least for anything other than very short-term exposures. We emphasize that the definition of target tissue dose surrogate used here is provisional and is likely to change as our understanding increases. In this regard, it is interesting to note that Laethem and Kedderis [4] found that 3-h incubations of freshly isolated rat and mouse hepatocytes with chloroform only affected hepatocyte viability measured at 24 h when chloroform concentrations were in excess of 3.8 mM. These data suggest that chloroform itself plays a direct role in the development of the hepatotoxic response, since rate of metabolism is maximal well below 3.8 mM. In future iterations of the quantitative model described here, the dose surrogate definition will be modified for consistency with this result.

3.4. Chloroform-induced regenerative replication

Larson et al. [5] have studied the induced cytotoxicity and cell proliferation in female B6C3F1 mice given chloroform by corn oil gavage over a wide dose range (0, 3, 10, 34, 90, 238, or 477 mg/kg) for either 4 consecutive days or 5 days/week for 4 weeks. In all cases, osmotic minipumps containing bromodeoxyuridine were implanted 3.5 days prior to sacrifice so that labelling index could be measured. Similar studies have been reported by Butterworth and coworkers for male B6C3F1 mice [6] and for male and female F344 rats [7,8]. The outstanding characteristics of these studies are (1) the wide range of doses used; (2) the use of repeated dosing; and (3) the use of osmotic pumps implanted for 3 days for measurement of cell division rates. Taken together, these characteristics lead to relatively high confidence in the quality of the data. Of particular interest in these studies is the presence of no-observed-adverse-effect-levels (NOAEL) for chloroform-induced regenerative replication. Such data are consistent with the presence of a dose threshold for cytolethality and consequent regenerative replication.

For the model described here, the cell replication data for female B6C3F1 mice and male F344 rats given chloroform for 4 days by corn oil gavage [5,7] were used. Future versions of the model will be developed with reference to all the data sets described above.

3.5. Linkage of the hepatic metabolism of chloroform with cytolethality and induced regenerative cellular replication

The TD submodel consists in part of an empirical function linking values of the dose surrogate, provided by the TK submodel, to the fraction of liver killed (Fig. 1). The amount of cell killing in the liver for given levels of chloroform exposure is inferred from the labelling index data by assuming a one-to-one correspondence between cells killed by chloroform and regenerative replications. Both the female B6C3F1 mouse liver and male F344 rat labelling index data were used to develop the linking

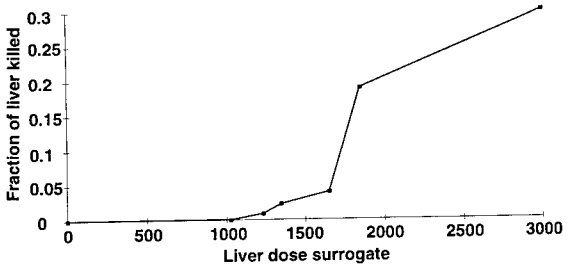


Fig. 1. Empirical function describing relationship between target tissue dose surrogate, as defined by the TK submodel, and cell killing inferred from labelling index data. During a simulated chloroform exposure, the value of the dose surrogate is calculated at each time point t, and the function specifies the corresponding amount of cytolethality. See the text for details.

function. This use of both the mouse and rat data required that the TK submodel be configured for the appropriate sex and species. This was done according to Corley et al. [1].

The TD submodel also contains a description of the cell kinetics of the hepatocyte population, allowing simulation of the labelling index. The submodel generates a basal labelling index due to the normal turnover of hepatocytes (number of cells dividing in an arbitrary interval/all cells) and simulates an increase in the labelling index when simulated regenerative replications occur. The TD submodel is configured to begin regenerative replication 48 h after chloroform-induced cytolethality first occurs. This delay is intended to approximate the time needed for the surviving cells to sense the killing of neighboring cells and begin the process of regenerative replication.

The combined TK-TD model provides good simulations of labelling index data (Fig. 2). For these simulations, the TK submodel was configured for either female B6C3F1 mice or male F344 rats according to Corley et al. [1]. The significance of good simulation of the labelling index data (Fig. 2) should not be overinterpreted, since the TD submodel was developed by fitting to these data. The test of the model comes in its ability to predict data obtained independently of the model development process. Future versions of the model described here will be tested thoroughly against such data. In the next section, we describe a preliminary attempt to model an episode of human chloroform poisoning.

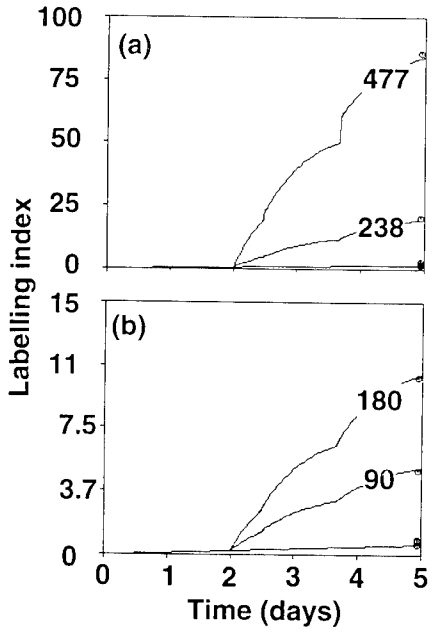


Fig. 2. Simulation of hepatic labelling index in (a) female B6C3F1 mice and (b) male F344 rats given chloroform by corn oil gavage for 4 consecutive days with sacrifice on day 5. Solid lines represent simulated labelling index. Numbers on lines represent chloroform doses (mg/kg/day). Data are represented by (○). Note the difference in scales for labelling index between mice and rats.

4. Preliminary simulation of the human hepatotoxicity of chloroform

Wallace [9] described a case of hepatitis and nephrosis in a 50-year-old man who consumed about 12 ounces/day of a cough syrup containing chloroform and 3% alcohol. After a prolonged period of cough syrup consumption at this rate, the individual experienced signs of both hepatic and renal toxicity. He had been consuming about 27 mg chloroform/kg/day. The TK submodel was configured for this individual according to Corley et al. [1], including the specification of his body weight at 78 kg, as reported by Wallace [9]. It is worth noting that Corley et al. [1] had used human liver microsomes to estimate the capacity of human hepatocytes to metabolize chloroform (V_{max} of 15.7 mg chloroform/kg body weight/h with scaling for different body weight by $BW^{0.7}$). With this configuration, a simulation of 7 days of cough syrup consumption showed no effect on labelling index, i.e. labelling index stayed at its basal value (Fig. 3). However, increasing V_{max} by a factor of 3 to 47.1 mg/kg/h resulted in an increase in simulated labelling index for the same exposure scenario. Under these conditions, the model was predicting significant chloroform hepatotoxicity. The change in V_{max} by a factor of 3 required to simulate liver damage does not seem unreasonable given (a) the coexposure to alcohol in the cough syrup and (b) the likelihood that prolonged exposure in this case may have caused

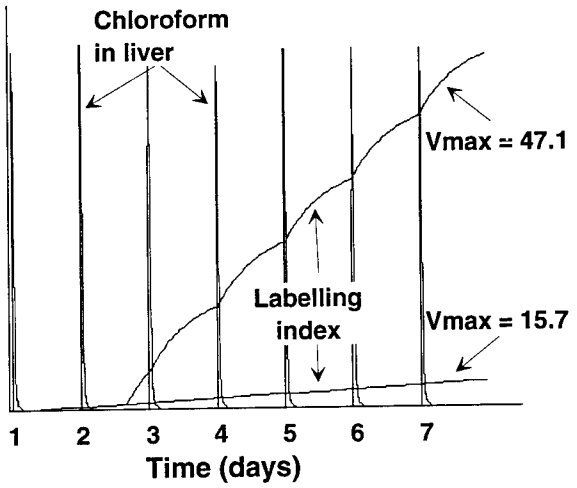


Fig. 3. Simulation of hepatic concentration of chloroform and hepatic labelling index in a 78-kg man consuming a bolus dose of 27 mg chloroform/kg/day for 7 days. Labelling index is increased when the capacity to metabolize chloroform (V_{max}) is set to 47.1 mg chloroform/kg body wt./day. With V_{max} at 15.7, the value obtained by Corley et al. [1], no increase in labelling index over the basal level was seen.

some induction of the capacity to metabolize chloroform. This result, while preliminary, suggests that the model development process to date is valid. However, more work is needed before the model can be used with confidence for quantitative prediction of the human hepatotoxicity of chloroform.

5. Discussion

Development of a preliminary, mechanism-based model for simulation of in vivo hepatocyte killing by chloroform has been described. The model consists of 2 submodels, one linking exposure with tissue dose (TK submodel) and one linking the tissue dose with toxic effect (TD submodel). The submodels overlap at the level of tissue dose of chloroform, since the tissue dose defined by the TK submodel is also the starting point for the TD submodel (Fig. 1). The overall model provides good descriptions of rodent hepatocyte labelling index data (Fig. 2) although this is expected since these data were used for model development. More interesting is the ability of the model, with some adjustment of the hepatic capacity for chloroform metabolism, to predict hepatotoxicity that occurred in a 50-year-old male consuming about 27 mg chloroform/kg/day [9] (Fig. 3).

The primary justification for development of the model must come from its potential for improving chloroform risk assessment. The current U.S. EPA noncancer risk assessment for chloroform is in the form of an oral reference dose (RfD) calculated from a study by Heywood et al. [10]. In this study, fatty cysts developed in beagle dogs given chloroform in a toothpaste base in gelatin capsules, 6 days/week for 7.5 years. The NOAEL for the study was 12.9 mg/kg/day, and the RfD was set at 0.01 mg/kg/day [11]. Uncertainty factors of 10 were applied to the NOAEL to account for interspecies conversion, protection of sensitive human subpopulations, and concern that the effect seen was a lowest-observed-adverse-effect-level (LOAEL) and not a NOAEL. The RfD was calculated without use of either TK or TD data specific to chloroform. In contrast, the mechanism-based, exposure-response model described here makes maximum use of available data to construct the TK and TD submodels. It seems reasonable to argue that predictions obtained with the mechanism-based model are less uncertain than is the RfD calculation. In this regard, it is interesting to note that the mechanism-based model predicts an hepatotoxic effect of 27 mg chloroform/kg/day in the human only when the metabolic capacity of human liver for chloroform is increased somewhat from the value obtained by Corley et al. [1].

Application of the mechanism-based model for chloroform in formal risk assessment should include use of Monte Carlo analysis to approximate variability in the human population. Confidence in the model could also be increased by more extensive use of human data for its parameterization. Studies designed to support these improvements are underway at the Chemical Industry Institute of Toxicology (CIIT). It will be possible, when these studies are complete, to use the model for prediction of human risk of hepatotoxicity from chloroform exposure. Use of an

uncertainty factor of perhaps 10 to account for residual model uncertainty should be sufficient to identify exposure levels reasonably expected to be without hepatotoxic effect in humans.

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Mechanisms in tumor promotion: guidance for risk assessment and cancer chemoprevention

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Abstract

In mouse skin, tumor development promoted by 'non-genotoxic' carcinogens is closely related to the wound response. In both cases endogenous factors such as cytokines and eicosanoids released primarily from 'activated keratinocytes' play a key role as mediators of inflammation and cellular hyperproliferation. The liberation of interleukin-1α and arachidonic acid from human keratinocytes has been used as an in vitro parameter of irritancy. The results (from experiments with 15 different chemicals) being validated at present in a clinical study indicate a quantitative relationship between irritancy in vivo and mediator release in vitro. In the course of experimental skin carcinogenesis an overproduction of eicosanoids due to a constitutive overexpression of the corresponding enzymes (i.e. PGH synthase-II and 8- and 12-lipoxygenase) is observed. Enzyme inhibitors, for instance nonsteroidal antiinflammatory drugs (NSAIDs), exert a strong tumoristatic effect. Thus, the approach of multistage skin carcinogenesis provides a suitable animal model for a mechanistic evaluation and further improvement of chemopreventive measures such as the inhibition of colorectal tumor development in humans by NSAIDs ('aspirin effect').

Keywords: Carcinogenesis; Interleukin-1α; Arachidonic acid metabolism; Irritancy; NSAID; Keratinocytes

1. Introduction

Cancer results from the progressive accumulation of genetic defects [1]. Nevertheless, a wide variety of genotoxic carcinogens is joined by a large number of so-called non-genotoxic carcinogens, i.e. agents that do not exhibit mutagenic potential in the commonly used tests [2]. A number of them are strong irritants and potent stimulators of cellular proliferation [3]. Non-genotoxic carcinogens are thought to facilitate the attack of genotoxic carcinogens (cocarcinogenesis) and to accelerate tumor development by selective processes, as well as by increasing the probability of additional oncogenic mutations and inducing the formation of endogenous mutagens in the course of permanent hyperproliferation and tissue inflammation (tumor promotion and progession).

Tumor promoters have been well defined on the basis of animal experiments. Together with genotoxic carcinogens they induce cancer in an

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extremely synergistic manner. The classical animal model for an investigation of such synergistic effects is provided by chemically induced skin cancer in mice [4,5]. In this model the highly irritant phorbol esters and related toxins have been identified as the most potent tumor promoters. By using these agents as experimental tools a rather deep insight into the molecular events of tumor development and of the tissue response to irritation was obtained. It turned out that phorbol esters and related tumor promoters evoke a tissue reaction which, down to the molecular details, resembles a wound response [6,7]. Also, mechanical wounding and non-specific irritation have been found to provide powerful tumor promoting stimuli in initiated tissue, indicating regenerative hyperproliferation and inflammation to be major factors in carcinogenesis [8].

Given that most skin tumors would remain latent in the absence of inflammatory hyperproliferation, skin cancer may indeed be regarded as the result of chronic irritation and 'permanently repeated and disturbed tissue regeneration' [9], as originally postulated by Rudolf Virchow. The critical role of tumor promoters and other irritants in carcinogenesis creates a serious toxicological problem which is still far from being solved. The inhibition of tumor promotion provides, on the other hand, a promising approach for cancer prevention.

2. Inflammatory hyperplasia and keratinocyte activation

A wound response may be understood as a coordinated repair and defence reaction which becomes visible as cellular hyperproliferation and inflammation (inflammatory hyperplasia). It results from an interaction between many different cell types and, therefore, depends on intercellular communication via signaling factors released from the cells involved. Such 'wound hormones' include growth factors, cytokines and lipid compounds acting as mediators of both mitogenesis and inflammation. In skin, such an inflammatory hyperplasia (hyperplastic transformation) is evoked not only by mechanical damage but by irradiation (UV) and chemical irritation as well [10], and the repeated induction of inflammatory hyperplasia represents a critical condition of tumor promotion [11].

While many chemicals induce inflammatory hyperplasia by unspecific tissue damage, other agents do so by interacting specifically with pathways of intracellular signal transduction. This group of irritants includes some of the most powerful skin tumor promoters such as the phorbol esters (interacting with protein kinase C and perhaps some other signaling proteins by mimicking the action of the second messenger diacylglycerol) and okadaic acid (interacting with phosphoprotein phosphatases). It may be concluded that these pathways of signal transduction are normally involved in the processing of signals delivered by the wound hormones mentioned above. Skin tumor promoters such as phorbol esters have been shown to mimic some primary effects of such mediators but also to evoke their release and biosynthesis, thus inducing the complex pattern of autocrine and paracrine interactions on which inflammatory hyperplasia depends.

In the skin, keratinocytes, the major cell type of the epidermis, seem to play a key role as a signaling interface between the environment and the organism and their activation by exogenous or endogenous stimuli is thought to provide a triggering event for skin inflammation and hyperplastic development [12,13]. In fact, activated keratinocytes have been shown to produce a complex cocktail of mediators, some of which react on keratinocytes along autocrine pathways, whereas others recruit and activate other cell types involved in the wound response. The mediators thus released form two interconnected networks of intercellular communication, i.e. the cytokine network and the eicosanoid network.

3. Interleukin-1α and eicosanoids as key mediators of inflammatory hyperplasia in skin

Among the various keratinocyte-derived cytokines [14,15], interleukin-1α (Il-1α) is thought to play a key role in the induction of skin inflamma-

tion and wound repair [13,16]. Non-activated keratinocytes are already synthesizing and accumulating this cytokine in extraordinarily high amounts [13]. Upon injury, Il-1α seems to be immediately released from these stores, probably as a result of cell destruction. Il-1α production in keratinocytes is induced in an autocrine manner by Il-1α [17], but also by growth factors, bacterial lipopolysaccharides and irritants as well as by thermal and mechanical injury and UVB-irradiation [12–20].

As a proinflammatory and immunological key mediator, Il-1α exhibits widespread systemic effects [21]. In skin Il-1α initiates the generation of a wide variety of other cytokines and growth factors [18], as well as the expression of intercellular adhesion molecules which mediate the interactions between keratinocytes and the cells of the inflammatory infiltrate [22].

Considering these manifold actions of Il-1α, it is not surprising that an intradermal injection of Il-1α has been found to induce an erythema and leukocyte infiltration in skin [23], that Il-1α was detected in skin exudates upon UV-irradiation, mechanical trauma and toxic insults [24], and that in mouse skin inflammatory hyperplasia induced by the tumor promoter TPA was inhibited by subcutaneous injection of an anti-Il-1α antibody [23]. Whether such a treatment would also suppress tumor development, as one might expect, is not known.

Eicosanoids represent the second family of proinflammatory mediators released from keratinocytes upon skin irritation (reviewed in [25]). Eicosanoids are not stored in cells and tissues, but synthesized on demand from polyunsaturated precursor fatty acids such as arachidonic acid, which are sequestered in cellular phospholipids. In the skin the cytosolic phospholipase A_2 ($cPLA_2$) of keratinocytes seems to provide the major enzyme for arachidonic acid release, being primarily responsible for the rapid eicosanoid accumulation in keratinocytes and skin in response to a wide variety of endogenous factors and exogenous stimuli. $cPLA_2$ activation occurs along several pathways of transmembrane signaling [26–29]. In addition, $cPLA_2$ expression [30,31] and eicosanoid formation [32] are induced by Il-1α, indicating an important link between the cytokine and the eicosanoid network [30,31]. In keratinocytes also a low-molecular weight secretory PLA_2 type II ($sPLA_2$ II) is induced by Il-1α and may contribute to the production of eicosanoids in skin (Fürstenberger et al., unpublished results).

Once released from phospholipids, arachidonic acid is metabolized by a series of oxygenases. Certain cytochrome P450-dependent monooxygenases yield epoxy derivatives as well as monohydroxy eicosatetraenoic acids (HETEs). Such an enzyme activity has also demonstrated for human keratinocytes [33]. Other enzymes, i.e. lipoxygenases, catalyze the formation of hydroperoxy metabolites (HPETEs), which are reduced to the corresponding hydroxy fatty acids (HETEs). 5-, 8-, 12- and 15-HETE as well as the leukotrienes A_4, B_4 and C_4 have been found in human and murine keratinocytes, and the expression of the corresponding lipoxygenases has been observed in epidermis and in keratinocytes (reviewed in [34]).

The third major pathway of arachidonic acid metabolism leads to the prostaglandins, such as PGE_2, $PGF_{2\alpha}$, and PGD_2, which have been found in skin preparations of several species. The main biosynthetic activity is localized in the epidermal layer and has been shown to be enhanced by a wide variety of stimuli, including Il-1α, TGF-α, phorbol esters, wounding and UV-light [34]. Two prostaglandin synthases, i.e. a constitutive PGHS-I and an inducible PGHS-II, have been characterized. PGHS-I is constitutively expressed in normal epidermis, whereas the expression of PGHS-II is strictly correlated with hyperplastic and neoplastic development [35,36].

Individual eicosanoids are involved in many steps of the acute inflammatory process, and prostaglandin synthesis has been found to provide a critical condition for keratinocyte mitogenesis in vivo [25] and in vitro [37].

4. Mediator release from keratinocytes as a parameter for toxicological testing

The extraordinary signaling capacity of kera-

tinocytes indicates a key role of this cell type in the induction of inflammatory hyperplasia and skin tumor promotion. Consequently, mediator release by keratinocytes may be considered to provide a suitable in vitro parameter for monitoring these tissue responses. Within the two signaling networks of eicosanoids and cytokines, arachidonic acid and Il-1α can be regarded as primary and arachidonic acid metabolites (eicosanoids) as secondary signals. Based on these considerations we set out to develop an in vitro irritation assay using the release of arachidonic acid and IL-1α from human keratinocytes (line HPKII) together with cell viability as endpoints.

Fifteen structurally unrelated compounds with graded irritant potential were tested. All of them induced a release of arachidonic acid and Il-1α, albeit with different kinetics and dose dependencies, indicating subtle differences in the mechanisms of action [38,39]. The reasons for these differences are not yet known. On the basis of the time courses, the test substances could be classified according to their ability to induce a rapid increase (i.e. within 1 h), a delayed increase (i.e. within 4–8 h), or a late increase (i.e. within 12–24 h) of mediator release. Inducers of a rapid arachidonic acid release were frequently, but not always, identical with rapid Il-1α inducers. Stimulation of arachidonic acid release generally occurred at lower concentrations of the test compounds than Il-1α release or at similar concentrations with more rapid kinetics, suggesting arachidonic acid release to be the more sensitive endpoint. The dose–response curves showed, however, that early inducers were not necessarily more potent than delayed or late inducers, thus indicating that a detailed kinetic analysis of the parameters is of critical importance for determining the potencies of the individual compounds. Evaluation of the half-maximal stimulatory concentrations (SC50) for arachidonic acid release and the tenfold-stimulatory concentrations (ED10) for Il-1α release allowed a ranking according to increasing potencies [38,39]. Although arachidonic acid release was found to be the more sensitive parameter when compared with the Il-1α-release for 11 out of 15 compounds, combined measuring of these endpoints led to a better grading of the irritant potencies of the chemicals. Independently of whether a rapid, delayed or late arachidonic acid release was induced, the dose-dependent ranking of the 15 compounds for their ability to induce this response was similar to, although not strictly identical with, the ranking of cell toxicity as measured by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. In several cases, arachidonic acid release occurred earlier or at lower concentrations of the test substance than an impairment of cell viability, indicating this response to precede rather than to follow cell damage. Il-1α release differed from arachidonic acid release in that it was evoked by 13 out of 15 chemicals at the same or higher concentrations as those required for 50% impairment of cell viability, indicating this response to result from damage, as postulated by other authors [13].

These results show that human keratinocytes grown in vitro indeed respond to chemicals of graded irritant potential with a graded release of proinflammatory key mediators and a graded impairment of viability. Therefore, a combination of these endpoints is expected to provide an in vitro assay of skin irritancy which could complete or even replace animal tests such as the Draize skin test. Unfortunately, the in vivo data found in the literature are of only limited value for a validation of the in vitro data. Being mainly based on rabbit Draize scores, they lack detailed evaluations of dose–response relationships and time-courses in most cases, not to mention the problems of interspecies differences. Therefore, we considered human in vivo data to be obligatory for a validation of the in vitro results.

For this purpose a placebo-controlled, open, randomized study using healthy volunteers (male caucasians, 18–45 years) is being carried out. In an initial screening phase vehicle solution and four concentrations of the test compounds were applied under occlusion onto the volar forearms of 20 volunteers (patch-test). Twenty-four, 48 and 72 h later transepidermal water loss was measured and the individual erythema dose was determined by visual scoring. By this means 12

responders were selected at random for the subsequent challenge phase which started 1–2 weeks later.

In the challenge phase vehicle solution and test chemicals (according to the individual erythema doses) were applied as described above. Six and 24 h later suction blisters were produced and the blister fluids were collected for mediator assays, either by enzyme immunoassay (Il-1α) or gas chromatography/NECI mass spectrometry (eicosanoids). While in cell cultures arachidonic acid released from cells is trapped by serum proteins, thus being protected from further metabolism, no such protection will occur in vivo. Therefore, a series of eicosanoids in addition to arachidonic acid was assayed in the blister fluids. These eicosanoids include PGD_2, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, LTB_4, 5-, 8-, 12-, and 15-HETE.

Results are at present available for three test compounds, i.e. benzalkonium chloride (BKCL), sodium dodecylsulfate (SDS) and triethanolamine (TEA). While BKCL and SDS rank among the most potent inducers of mediator release in vitro, TEA was about four orders of magnitude less active in this respect (Fig. 1). The erythema assay in vivo led to a

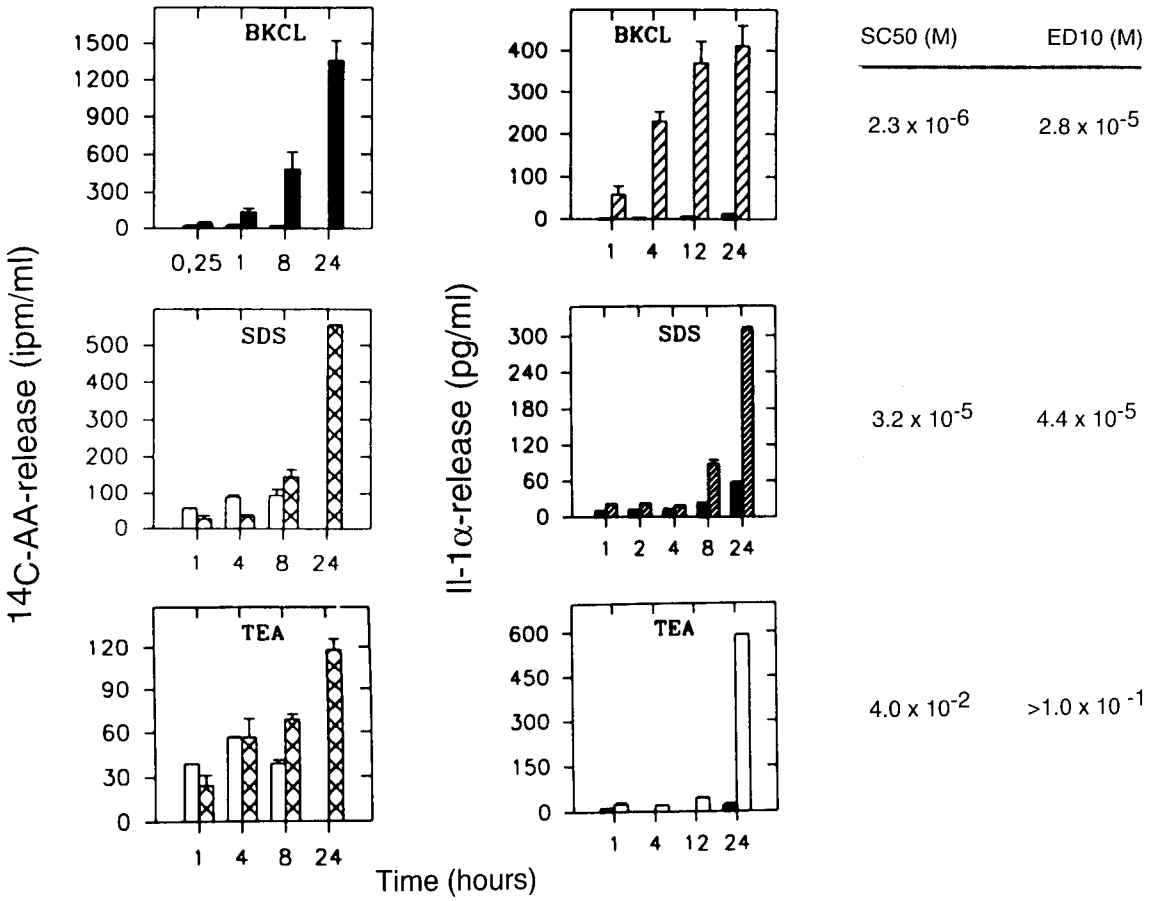


Fig. 1. Response of HPKII human keratinocytes in vitro to benzalkonium chloride (BKCI, 50 μM for Il-1α release, 20 μM for arachidonic acid release), sodium dodecylsulfate (SDS, 100 μM), and triethanolamine (TEA, 100 mM). The release of [^{14}C]arachidonic acid (from prelabelled cells) and of Il-1α into the culture medium was measured at the times indicated. The vehicle controls are represented by open bars (arachidonic acid release) or black bars (Il-1α release). On the right side the half maximal stimulatory concentrations (SC 50) for arachidonic acid release and the tenfold-stimulatory concentration (ED 10) for Il-1α release are shown. For details see [39].

comparable result. As shown in Fig. 2, BKCL and SDS evoked a response in the majority of the test persons whereas only 1–2 out of 12 volunteers exhibited a weak reaction upon TEA application. Moreover, the response to BKCL developed more rapidly than that to SDS, thus corresponding even in this detail to the responses of keratinocytes in vitro. The results of the mediator assays (not shown) support the data of the in vitro assays in that the majority of the test persons treated with irritants exhibited elevated concentrations of Il-1α and the above-mentioned eicosanoids as compared with the vehicle controls. Depending on the metabolite, 1.2- to 40-fold elevations were observed. It must be emphasized, however, that the mediator assay is of rather limited value since the production of suction blisters alone provides a strong irritant effect. As a consequence considerable amounts of mediators were found in the blister fluids of the vehicle controls, and the mediator concentrations were subject to large individual variations.

Our results indicate that irritation of human skin apparently corresponds to the Il-1α and arachidonic acid release from human keratinocyte cultures. Therefore, these parameters may be suitable for the development of a novel in vitro test which is based on mechanistic considerations and thus expected to provide considerably more information than a conventional cytotoxicity test [40]. It must not be overlooked, however, that an in vitro test based solely on keratinocytes certainly represents an oversimplification, since other cell types of the skin may participate in the release of primary proinflammatory mediators as well, perhaps depending on the type of the challenge.

Would it be possible to use such an in vitro test also for a screening of tumor promoters? As far as skin tumor promoters are concerned, such a possibility may well be envisaged, since the great

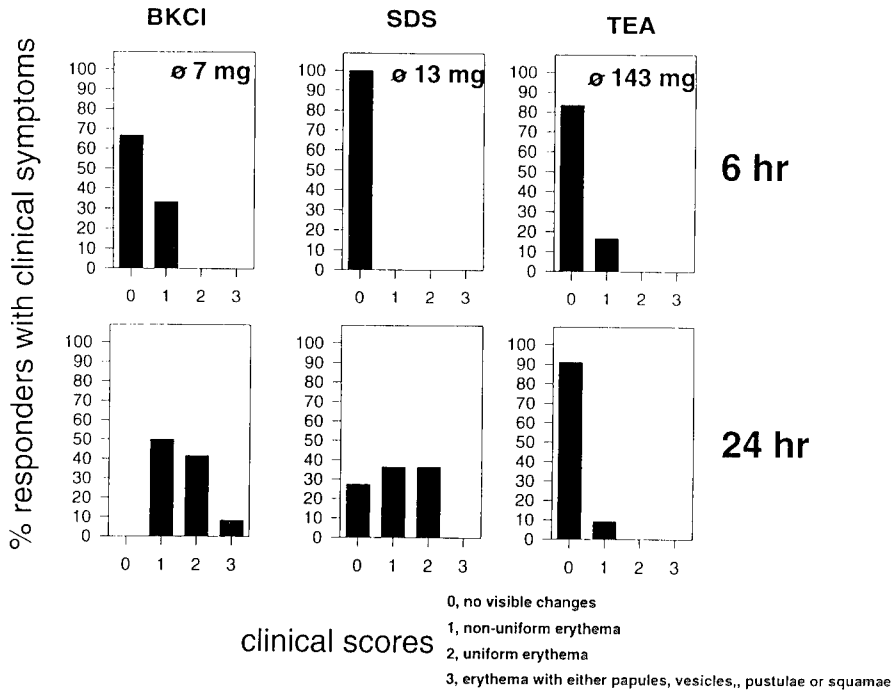


Fig. 2. Response of human skin in vivo to topical applications of benzalkonium chloride (BKCI), sodium dodecylsulfate (SDS), and triethanolamine (TEA). Aqueous solutions of the substances were applied under occlusion on the volar forearm skin of 12 volunteers each at zero time and the responses were evaluated at the times indicated. Clinical score 0, no change; 1, weak non-uniform erythema; 2, weak uniform erythema; 3 erythema, papules, vesicles. The mean individual doses (according to an initial screening) were 7 mg BKCI, 13 mg SDS, and 142 mg TEA. Ordinate: number of responders (%).

majority of them are indeed irritants, which induce inflammatory hyperplasia. It is not yet known whether such a close relationship between irritating and tumor promoting potential holds true also for tumor promoters acting on other organs. On the other hand, a number of non-genotoxic carcinogens have been found to be strong inducers of inflammation and hyperproliferation in their target tissues [3]. It must be emphasized, however, that for unknown reasons not every agent or manipulation that induces eicosanoid formation and inflammatory hyperplasia in mouse skin is a potent tumor promoter [5,6]. Perhaps such non-promoting irritants interrupt tumor development at another stage or induce desensitization of the tissue in the course of the chronic treatment that is required for tumor promotion.

5. Arachidonic acid metabolism as a target of tumor chemoprevention

In the mouse skin model both inflammatory hyperplasia and tumor promotion have been shown to depend critically on the local formation of arachidonic acid metabolites [25,34]. Thus, the initial induction of epidermal hyperplasia depends on the rapid release of PGE_2 (within 10 min after treatment), most probably from keratinocytes, whereas chronic hyperplasia and tumor promotion are strictly $PGF_{2\alpha}$-dependent [41]. In the course of tumor development a strong accumulation of both prostaglandins is observed in neoplastic but not in non-neoplastic skin (Table 1 and [35]). In normal (untreated) epidermis only prostaglandin H synthase I (PGHS-I) is found, whereas PGHS-II expression is rapidly but transiently induced during hyperplastic transformation [35,36]. In benign and malignant epidermal tumors a constitutive overexpression of PGHS-II is observed, whereas the level of PHGS-I remains unchanged [35]. It may be concluded, therefore, that the initial PGE_2 synthesis required for the induction of inflammatory hyperplasia is catalyzed by PGHS-I whereas the prostaglandin accumulation found in neoplastic skin results from the overexpression of PGHS-II. Most probably, the constitutive PGHS-II expression in tumors occurs via an autocrine pathway, since growth factors such as TGFα and cytokines such as Il-1α, which are known to induce PGHS-II expression, have been found to be released by activated and neoplastic keratinocytes.

Local treatment of mouse skin with PGHS inhibitors such as indomethacin counteracts the effects of tumor promoters, i.e. prevents local prostaglandin accumulation and epidermal hyperplasia and brings tumor development to a halt [25,41]. Since at least in the early phases of tumorigenesis the (still benign) epidermal tumors grow in a reversible manner [5], such a treatment results in tumor regression and prevention of malignant progression. This tumoristatic effect of indomethacin can be specifically overcome by application of $PGF_{2\alpha}$, indicating this prostaglandin to play a key role in skin tumor development, probably as a mediator of sustained hyperplastic

Table 1
Tissue levels of eicosanoids in the course of multistage carcinogenesis in NMRI mouse skin

	Untreated epidermis	Acute hyperplasia[a]	Chronic hyperplasia[b]	Papilloma	Carcinoma
PGE_2	35	85	40	720	800
$PGF_{2\alpha}$	0.5	4	2	15	18
5-HETE	21	36	29	19	1
8-HETE	71	205	789	2764	66
12-HETE	43	57	209	3963	628
15-HETE	74	172	158	322	41

Tumors were generated according to the inltiation–promotion protocol with 7,12-dimethylbenz[a]anthracene as initiator and phorbol ester TPA as promoter [35]. The prostaglandin levels were determined by enzyme immunoassay [39] and are given in ng/mg protein. The HETE levels were assayed by GC/MS and are given in ng/g tissue [50]. $N \geq 4$, S.D. $\leq 25\%$.

[a] Assayed 6 h (prostaglandins) or 24 h (HETEs) after a single topical application of 10 nmol TPA.

[b] Assayed 2 weeks after 36 applications of 10 nmol TPA, in 3 day intervals.

transformation [41]. In addition, prostaglandin metabolism may exert other co-carcinogenic effects such as an activation of chemical carcinogens, production of mutagenic malondialdehyde or an impairment of immunological defence mechanisms [42], as well as thromboxane A_2-dependent promotion of metastasis [43].

Does the multistage approach of mouse skin tumorigenesis provide a model for human tumor development? It is a well-established fact that human tumors also progress in a stepwise manner with the discrete stages representing distinct patterns of genetic alterations. This has been convincingly shown for colorectal carcinogenesis [1]. Actually, this tumor type has become the subject of chemoprevention by PGHS inhibitors such as aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs) (summarized in [42,44]). In a series of studies it has been shown that a chronic intake of aspirin correlates with an up to 50% reduction of the risk to become affected by or to die of colorectal cancer. Moreover, treatment of patients suffering from familial adenomatous polyposis, a hereditary precancerous condition, has been found to result in an almost complete tumor regression and in a corresponding reduction of the risk of malignant progression [45]. According to these observations, NSAID treatment ranks among the most powerful chemopreventive measures known at present. These promising epidemiological and clinical results are supported by a series of animal experiments indicating the promotion stage of large bowel carcinogenesis to be involved in the preventive action of NSAIDs [42,46]. According to the mouse skin model PGHS-II-catalyzed prostaglandin synthesis probably would provide the target proper of NSAID-based chemoprevention. Recently, an overexpression of PGHS-II has been found to correlate also with colorectal tumor development in humans [47]. These results indicate that PGHS-II-specific inhibitors would be superior to the conventional NSAIDs, which inhibit both PGHS isoenzymes and produce more side-effects than PGHS-II inhibitors [48]. Moreover, the mouse skin model indicates that chemoprevention by NSAIDs is a more general phenomenon, which may not be restricted to colorectal cancer but – depending on the type of drug and the regimen of application – be applied to other tumor types as well.

Finally, the mouse skin model may be used as a guide for the development of novel preventive and therapeutic measures. Using this model we have found not only the PGHS-dependent branch but also the lipoxygenase-dependent branch of arachidonic acid metabolism to become overactivated in the course of tumor development. This overactivation results in a dramatic accumulation of hydroxyeicosatetraenoic acids such as 8- and 12-HETE, in particular in premalignant papillomas (Table 1 and [49]), which correlates with an overexpression of the corresponding lipoxygenase activities [50]. Similarly to PGHS-II, these lipoxygenases are transiently induced upon hyperplastic transformation, but become constitutively overexpressed in neoplastic tissue. The specific inhibition of these enzymes by low doses of ETYA results in a strong antineoplastic effect comparable to that observed for NSAIDs [51]. These results indicate that skin tumor development depends on the overexpression of both PGHS-II and lipoxygenases. Since 8- and 12-HETE as well as their hydroperoxy precursors induce chromosomal damage in keratinocytes (Fig. 3), arach-

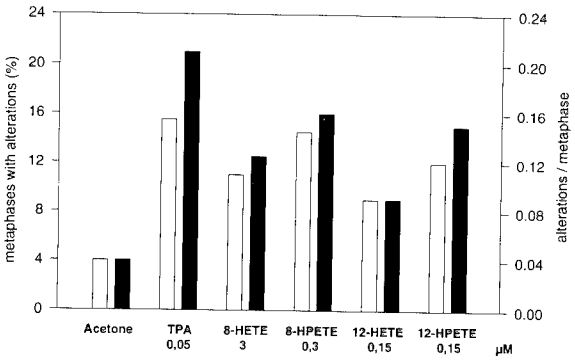


Fig. 3. Clastogenic effects of the tumor promotor TPA and of 8- or 12-HETE/HPETE on primary neonatal mouse keratinocytes in vitro. The cells were treated with the concentrations indicated and analyzed for metaphases with chromosomal alterations (left ordinate, empty columns) and number of alterations per metaphase (right ordinate, filled columns). The experiment was carried out according to [51].

idonic acid metabolism may be involved not only in tumor promotion but also in the progression to malignancy. After all, in the mouse skin model malignant progression of papillomas occurs spontaneously, i.e. without additional treatment, indicating endogenous mutagenic factors to be involved. It has been postulated that oxidative DNA damage induced by endogeneously produced active oxygen species, peroxy compounds and organic free radicals provides a major mechanism of carcinogenesis [8]. Arachidonic acid metabolism and inflammatory processes mediated by arachidonic acid metabolites are certainly rich sources of such genotoxic agents and other mitogens [52–54].

Provided an overexpression of lipoxygenase-catalyzed arachidonic acid metabolism is also found in human tumors and premalignant lesions, a specific inhibition of the corresponding enzymes is expected to provide a novel target for chemoprevention that would complement the effects of other treatments such as application of NSAIDs and intake of antioxidants [8].

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Understanding mechanisms of inhaled toxicants: implications for replacing default factors with chemical-specific data

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Abstract

Assessing risk of inhaled materials is a challenging endeavor because of the profound interspecies differences in respiratory tract anatomy, physiology, and biochemistry. Recent advances in the availability of mechanistic data and mathematical models for describing dosimetry behavior of particles and gases has lead to improvements in default approaches to risk assessment of inhaled materials. An overview of some of the more well-understood differences between species in factors controlling dosimetry and response, and the default approach of the U.S. Environmental Protection Agency that accounts for many of these factors, are presented. The default methodology also creates a framework which inhalation toxicologists can use to direct research at reducing uncertainty in risk assessments that might otherwise be handled through default uncertainty factors. The optimal approach to risk assessment is to develop chemical-specific mode of action and dosimetry data that can be used quantitatively to replace the entire default approach. The toxicology of vinyl acetate and recent efforts to develop data to supplant assumptions made in the default approach are presented. The conclusion is drawn that the future of inhalation toxicity risk assessment lies in reducing uncertainties associated with interspecies extrapolation and that to do this effectively requires approaches to toxicology that are outside of routine testing paradigms, and are aimed at elucidating mechanisms of action through hypothesis-driven research.

Keywords: Nose; Lung; Inhalation toxicity; Risk assessment; Dosimetry modeling

1. Introduction

Chemical dose-response assessments have for years attempted to adequately account for a variety of factors that contribute to interspecies differences in chemical-induced toxicity. The toxicology of the respiratory tract is a particularly challenging subject for risk assessors owing to the many anatomical, physiological, and biochemical differences between test species and humans. The nasal cavity, despite being positioned as the first potential site of deposition and reaction for inhaled substances, has been studied extensively only since the early 1980s [1]. Such studies have lead to an appreciation of the significance of portal-of-entry effects in this re-

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gion. Generally, the state-of-the-science of contemporary bioassays is now such that much more mechanistic data are available for toxicological assessment. Concurrently, mathematical models have become useful to characterize interspecies dosimetry[1] differences as well as dose-response functions. These models are significantly reducing the uncertainty associated with human health estimations derived from rodent toxicity data. This review article summarizes briefly, and superficially in many cases, how mechanistic data can be brought to bear on the difficult subject of comparative risk assessment of respiratory tract toxicants. Emphasis is placed on how flexible approaches, such as the U.S. Environmental Protection Agency's methods for derivation of inhalation reference concentrations, provide a framework that allows for iterative application of mechanistic data to dose-response assessment and can direct data gathering to address areas of uncertainty and default assumptions.

2. Factors controlling comparative inhaled disposition

The various species used in inhalation toxicology studies that serve as the basis for dose-response assessment do not receive identical doses in a comparable respiratory tract region (extrathoracic, ET; tracheobronchial, TB; or pulmonary, PU) when exposed to the same aerosol or gas [2]. Such interspecies differences are important because the adverse toxic effect is likely more related to the quantitative pattern of deposition and subsequent clearance and redistribution than to the exposure concentration. This section describes the major factors that control comparative inhaled dose. Detailed description of such factors is beyond the scope of this short review and the reader is referred to several extensive discussions of particle deposition [3–6] and gas absorption [3,6–10] in the respiratory tract. Although the various factors are discussed as distinct entities, it is important to appreciate that the factors influencing chemical disposition are dynamic and interactive, thus an accurate description of the processes requires integration. These factors are in turn influenced by exposure concentration, duration, and frequency.

2.1. Particles

Particles are deposited in the respiratory tract by mechanisms of impaction, sedimentation, interception, diffusion, and electrostatic precipitation. Subsequent clearance of a deposited dose is dependent on the initial site of deposition, the physicochemical properties of the particles (e.g., solubility), and on the time since deposition. Clearance routes include dissolution into respiratory tract tissues, absorption into the blood, removal to the gastrointestinal tract via the nasopharynx or mucociliary escalator, endocytosis by macrophages or epithelial cells, and absorption into the lymphatic channels.

2.2. Gases

The major processes affecting gas transport involve convection, diffusion, absorption, dissolution, and chemical reactions. The bulk movement of an inhaled gas in the respiratory tract is induced by a pressure gradient and is termed convection. Convection can be broken down into components of advection (horizontal movement of a mass of air relative to the airway wall) and eddy dispersion (air mixing by turbulence so that individual fluid elements transport the gas and generate flux). Molecular diffusion is superimposed at all times on convection due to local concentration gradients. Absorption removes gases from the lumen and affects concentration gradients. Chemical reactions in the respiratory tract can increase absorption by acting as a sink to drive the concentration gradient. Systemic metabolism can also drive the concentration gradient for insoluble gases that are removed from the respiratory tract tissue by perfusion. Thus, the rate of transfer from the environment to the tissue, the capacity of the body to retain the material, and elimination of the parent and metabolites by chemical reaction, metabolism, exhalation, and excretion influence the disposition of gases.

[1] Dosimetry modeling is used as a more comprehensive term than physiologically based pharmacokinetic (PBPK) modeling to capture not only model structures used to address volatile organic chemicals but also irritant gases and particles.

2.3. Physicochemical characteristics

For a given aerosol, the 2 most important parameters determining deposition are its mass mean aerodynamic diameter (MMAD) and the distribution of the particles about the mean. MMAD can be affected by the hygroscopic nature of some particles. As water is absorbed, the density and dimensions of the particle change. Solubility of particles influences their dissolution and clearance. For gases, the properties of water solubility and reactivity influence their interaction with the respiratory tract and uptake. Reactivity includes both the propensity for dissociation and the ability to react either spontaneously or via enzymatic reaction in the respiratory tract. Systemic metabolism, perfusion rates, and elimination rates modulate the uptake of insoluble gases.

2.4. Anatomic factors

Anatomic differences among species, especially rodents and humans, are important factors controlling interspecies differences in regional toxicity of inhaled materials. Species differences in gross anatomy, nasal airway epithelia (including type and location), and the distribution and composition of mucous secretory products have been noted [11,12]. Differences in structure of the upper respiratory tract (URT) and resultant differences in airflow dynamics and lesion distribution have been described [13–15]. In general, quadruped species such as rodents and dogs have a very complicated nasal turbinate structure, which increases airflow turbulence, and highly developed olfactory systems. On the other hand, primates, including humans, have relatively poorly developed turbinate structures and greatly reduced olfactory systems. To illustrate the latter difference, approximately 50% of the total surface area of the rat nasal cavity is lined with olfactory epithelium while this figure is approximately 10% for humans [13,16]. The effect of turbinate structure on URT deposition of vapors can be illustrated by comparative studies with acetone which is considered a relatively nonreactive, and poorly metabolized vapor. In a data set summarized by Morgan and Monticello [17], acetone deposition efficiency in the rat, guinea pig, dog and human was 12–45%, 7–20%, 52–60%, and 18%, respectively. The highest rates of deposition were found in the dog which, among the species compared, has the most complex set of anterior turbinates.

There are also clear differences between species with regard to the anatomy and geometry of the lower airway (for recent reviews, see [18,19]) that influence deposition and uptake of inhaled substances. The most obvious difference between rodents and humans is the branching pattern of the bronchi and bronchioles of the lung. In rodents, the branching pattern is asymmetric which presents a relatively unimpeded flow of inspired air to the lower respiratory tract. The branching pattern in humans, on the other hand, is symmetric presenting an airflow pattern more susceptible to deposition at branch points. Airway dimensions such as length and diameter are also different across species.

As in the URT, other anatomical differences between rodents and humans include epithelial cell types and numbers. While in the rat the predominant cell types lining the trachea are serous and ciliated cells, the predominant cell types in bonnet monkeys, are ciliated and basal cells. The distal airways of humans contain several generations of nonrespiratory bronchioles, followed by several generations of respiratory bronchioles and alveolar ducts. In contrast, the terminal bronchioles of rodents are generally contiguous with corresponding alveolar ducts [18]. The differences in cell types populating the alveolar regions are less dramatic. There appears to be a significant homogeneity in populations of epithelial, endothelial, interstitial, and macrophage cells of the alveolar regions and in the percentage of alveolar surface area covered by Type I and Type II cells [18,20]. Thus, toxicants that target the cell types or anatomical features that are specific to an individual species are likely to present difficulties for interspecies extrapolation of dosimetry and response.

2.5. Physiological factors

One of the most obvious and significant physiological differences between rodents and

humans is the fact that humans are oronasal breathers while rodents are obligate nose breathers. This has important ramifications for particle and gas deposition in the upper airways of humans as oral breathing increases with exertion [21]. Both particles and gases can escape the scrubbing effect of the URT under these conditions resulting in a greater delivery of material to the peripheral airways.

Differences in ventilation rates affect the tidal volume and ventilation-to-perfusion ratios across species. Cardiac outputs and tissue volumes also vary. These differences are important factors that interact with the anatomic differences described above to result in dramatic differences in deposition and uptake across species.

Mechanisms for clearing particles from the TB region are largely dependent on dissolution and physical clearance of poorly soluble materials by transport to the digestive tract along the mucociliary escalator or removal by macrophages and epithelial cells following phagocytosis. Clearance in the PU is accomplished by dissolution, absorption into the blood or lymphatic channels, and macrophage-mediated removal. In fact, chemotactic attraction of macrophages shows interspecies differences in response. For example, rat macrophages respond best to complement-derived chemotactic factors whereas hamster-derived macrophages respond best to *N*-formyl peptides [22]. As a result, rats have a greater capacity to clear materials such as carbonyl iron. These data raise the question of what differences arise with human macrophage clearance characteristics. Unfortunately, these are areas not yet addressed by quantitative research and can only be considered qualitatively when evaluating toxicity data.

2.6. Biochemical factors

Interspecies differences in biochemical mechanisms of airway activation, detoxication, and responses to inhaled toxicants are largely uncharacterized and present many opportunities for reducing uncertainties in risk assessments of chemicals that undergo metabolic activation or detoxication. Interspecies comparison of metabolic capabilities of the nasal cavity was recently completed [23,24] which shows that cytochrome P450 activities for a variety of substrates are metabolized less efficiently in microsomes from human nasal mucosa than that of rodents. On the other hand phase II enzymes, such as nasal tissue epoxide hydrolase and glutathione *S*-transferases, appear to be less active in rodents than in humans. A variety of other non-cytochrome P450 xenobiotic-metabolizing enzymes have also been identified and localized within specific cell types of the nasal epithelium [25], but interspecies differences in the activity of these enzymes is largely unknown. Carboxylesterase activity is particularly prominent in the nasal cavity of rodents [25–27]. Research on the carboxylesterase-mediated metabolism of esters suggest that humans have a reduced capacity compared to rodents [28,29].

With regard to the lower airways, comparative data on metabolic capabilities are also sparse. Tissue content and activities of cytochrome P450 and glutathione *S*-transfereases in human lung are generally lower than most species and considerably lower than that of mouse [30,31]. These results suggest significant differences exist in both phase I and phase II xenobiotic-metabolizing enzymes. Chemical- and mechanism-specific knowledge of these differences could contribute to reductions in uncertainties in risk assessments.

3. Default dose-response approach

The methods to derive inhalation reference concentrations (RfCs) are based on the same conceptual approach as that of the oral reference dose (RfD). There is one major exception. The RfC methods incorporate a dosimetric adjustment factor (DAF_r) for either respiratory tract region effects or remote toxicity [3]. The DAF_r is used to adjust for species differences in dosimetry. It is a multiplicative factor that represents the laboratory animal to human ratio of a given dose and is applied to laboratory animal exposure effect levels to calculate the human equivalent concentration (HEC). The HEC is expected to be associated with the same delivered dose to the observed target tissue as in the experimental species. The DAF_r calculated depends on (1) the physicochemical characteris-

tics of the inhaled toxicant (particle or 1 of 3 gas categories), (2) the location of the observed toxicity (i.e., either 1 of 3 respiratory tract regions or at remote sites), and (3) the type of dosimetry model (default or optimal) available for a particular chemical [3,32].

The derivation of an RfC from laboratory animal data is generally as follows. First, because many inhalation toxicity studies using laboratory animals are intermittent exposure regimens, a concentration (C) times time (t) product ($C \times t$) prorate adjustment is used to normalize these exposures to a continuous exposure as

$$\mathrm{NOAEL}^{*}_{[\mathrm{ADJ}]}\ (\mathrm{mg/m^3}) = E\ (\mathrm{mg/m^3}) \times D\ (\mathrm{h/day/24\ h}) \times W\ (\mathrm{days/7\ days}) \quad (1)$$

where the $\mathrm{NOAEL}^{*}_{[\mathrm{ADJ}]}$ is the no-observed-adverse-effect level (NOAEL) or analogous effect level obtained with an alternate approach such as the benchmark dose approach, which is adjusted for duration of experimental regimen; E is the experimental exposure level; D is the number of (h exposed/day)/24 h; and W is the number of days (days of exposure/week)/7 days. The above duration adjustment is also applied to lowest-observed-adverse-effect levels (LOAELs).

The benchmark dose approach has been proposed as an improvement on the NOAEL/LOAEL approach. In general terms it is the use of a specific mathematical model (e.g., Weibull, logistic, polynomial) to determine a concentration associated with a predefined response (e.g., 10% response of a dichotomous outcome) and an estimate of its lower bound. This approach has a number of advantages in that estimates derived take into account the slope of the exposure-response curve and the statistical rigor of experimental design. Guidance on the application of this approach to derivation of RfD and RfC estimates is presented elsewhere [33,34]. The use of the benchmark dose approach does not obviate the requirement for uncertainty factors (UFs) with the exception of the UF for LOAEL to NOAEL extrapolation.

The rationale for the default duration adjustment is that the resultant continuous human exposure concentration should be the ($C \times t$) equivalent of the laboratory animal exposure level. An advantage of dosimetry models is that because they incorporate and integrate various physicochemical and physiological determinants of chemical disposition, and thus dynamically simulate intermittent or continuous exposure, the use of this duration adjustment is obviated. Consideration of the basis of this adjustment is beyond the scope of this presentation and has been reviewed elsewhere [35].

The RfC methods then calculate the HEC by applying a $\mathrm{DAF_r}$ to the laboratory animal exposure effect level in order to account for species differences in dosimetry as

$$\mathrm{NOAEL}^{*}_{[\mathrm{HEC}]}\ (\mathrm{mg/m^3}) = \mathrm{NOAEL}^{*}_{[\mathrm{ADJ}]}\ (\mathrm{mg/m^3}) \times \mathrm{DAF_r} \quad (2)$$

where the $\mathrm{NOAEL}^{*}_{[\mathrm{HEC}]}$ is the NOAEL human equivalent concentration; $\mathrm{NOAEL}^{*}_{[\mathrm{ADJ}]}$ is defined in Eq. (1); and $\mathrm{DAF_r}$ is a dosimetric adjustment factor for either an effect in a specific respiratory tract region, r (ET, TB, PU, or TOTAL) or remote effects. The $\mathrm{DAF_r}$ is either the regional deposited dose ratio (RDDRr) for particles or the regional gas dose ratio (RGDRr) for a given gas category and type of effect [3]. The $\mathrm{DAF_r}$ is constructed using default normalizing factors for the physiological parameters of interest. For example, because insoluble particles deposit and clear along the surface of the respiratory tract, the deposited dose in a specific region (e.g., TB) is commonly normalized to the surface area of that region. Extrarespiratory or remote effects are usually normalized to body weight.

Once the HEC is calculated, the UFs as shown in Table 1 are applied (as required) to calculate the RfC as

$$\mathrm{RfC} = \mathrm{NOAEL}^{*}_{[\mathrm{HEC}]}/(\mathrm{UF} \times \mathrm{MF}) \quad (3)$$

The UFs are generally an order of magnitude, although incorporation of dosimetry adjustments or other mechanistic data has routinely resulted in the use of reduced UFs for RfCs. The composite UF applied to an RfC will vary in magnitude depending on the number of extrapolations required. An RfC will not be derived when use of the available data involve greater than 4 areas of extrapolation. The composite UF when 4 factors are used is reduced from 10 000 to 3000

Table 1
Guidelines for the use of UFs in deriving inhalation RfC

Standard UFs	Processes considered in UF purview
H, human to sensitive human Use ≤ 10-fold factor when extrapolating from valid experimental results from studies using prolonged exposure to average healthy humans. This factor is intended to account for the variation in sensitivity among the members of the human population.	Pharmacokinetics/pharmacodynamics Sensitivity Differences in mass Activity pattern Does not account for idiosyncrasies
A, laboratory animal to human Use ≤ 3-fold factor when extrapolating from valid results of long-term studies on experimental animals when results of studies of human exposure are not available or are inadequate. This factor is intended to account for the uncertainty in extrapolating animal data to the case of average healthy humans. Use of a 3 is recommended with default dosimetric adjustments. More rigorous adjustments may allow additional reduction. Conversely, judgment that the default may not be appropriate could result in an application of a 10-fold factor.	Pharmacokinetics/pharmacodynamics Relevance of laborabory animal model Species sensitivity
S, subchronic to chronic Use ≤ 10-fold factor when extrapolating from less than chronic results on experimental animals or humans when there are no useful long-term human data. This factor is intended to account for the uncertainty in extrapolating from less than chronic NOAELs to chronic NOAELs.	Accumulation of chemical or cumulative damage Pharmacokinetics/pharmacodynamics Severity of effect Recover Duration of study Dependence of effect on duration.
L, $LOAEL_{(HEC)}$ to $NOAEL_{(HEC)}$ Use ≤ 10-fold factor when deriving an RfC from a $LOAEL_{(HEC)}$ instead of a $NOAEL_{(HEC)}$. This factor is intended to account for the uncertainty in extrapolating from the $LOAEL_{(HEC)}$s to $NOAEL_{(HEC)}$s.	Severity Pharmacokinetics/pharmacodynamics Slope of dose-response curve Relationship of endpoints Functional vs. histopathological evidence
D, incomplete to complete database Use ≤ 10-fold factor when extrapolating from valid results in experimental animals when the data are 'incomplete'. This factor is intended to account for the inability of any single animal study to adequately address all possible adverse outcomes in humans.	Lack of second species Data gaps (lack of potential end points) Comprehensiveness of critical and supporting studies Exposure uncertainties
Modifying factor (MF) Use professional judgment to determine whether another uncertainty factor (MF) that is ≤ 10 is needed. The magnitude of the MF depends upon the professional assessment of scientific uncertainties of the study and database not explicitly treated above (e.g., the number of animals tested or quality of exposure characterization). The default value for the MF is 1.	

Source: Refs. 3 and 45.

in recognition of the lack of independence of these factors. The lack of independence is evident in Table 1 which shows the various pharmacokinetic and pharmacodynamic processes typically believed to be encompassed by each UF.

By definition, a database for derivation of a dose-response estimate for noncancer toxicity should ensure that both appropriate and

adequate numbers of endpoints have been evaluated. The minimum requirement for derivation of an RfC with low confidence is a well-conducted subchronic inhalation bioassay that evaluated a comprehensive array of endpoints, including an adequate evaluation of respiratory tract effects, and established an unequivocal NOAEL and LOAEL. Chronic inhalation bioassay data in 2 different mammalian species, developmental studies in 2 different mammalian species, and a 2-generation reproductive study may be required to establish high confidence. The rationale supporting these data requirements is that, since the objective of the RfC is to serve as a lifetime estimate, all potential endpoints at various critical life stages must be evaluated. Well-defined and conducted subchronic toxicity studies are considered to be reliable predictors of many forms of chronic toxicity, with the notable exceptions of carcinogenic, teratogenic, and reproductive effects. The specific requirement for adequate respiratory tract evaluation arises from the increased potential for the portal-of-entry tissue to interact intimately with chemicals. Dosimetry data that indicate distribution to extrarespiratory tract sites is insignificant (e.g., a highly reactive and irritant gas which causes respiratory tract damage) may obviate the requirement for reproductive and developmental data. If these minimum database requirements are not met, an RfC is not derived.

4. RfC framework for interspecies dosimetry adjustments

As illustrated in Fig. 1, it is ultimately desirable to have a comprehensive biologically based dose-response model that incorporates the mechanistic determinants of chemical disposition, toxicant-target interactions, and tissue responses integrated into an overall model of pathogenesis in order to characterize the exposure-dose-response continuum. Unfortunately, the data to construct such comprehensive model structures do not exist for the majority of chemicals. Without dosimetry, default methods for dose-response estimation are limited to the rudimentary ('black-box') level and necessarily incorporate

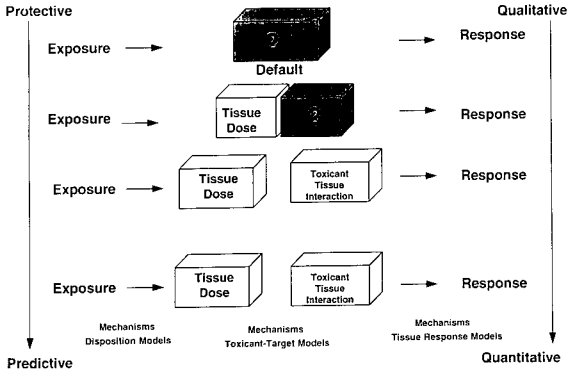


Fig. 1. Schematic characterization of comprehensive exposure-dose-response continuum and the evolution of protective to predictive dose-response estimates [3].

large UFs to ensure that the estimates are protective in the presence of substantial data gaps. With each progressive level, incorporation and integration of mechanistic determinants allow elucidation of the exposure-dose-response continuum and, depending on the knowledge of model parameters and fidelity to the biological system, a more accurate characterization of the pathogenesis process. Due to the increase in accuracy of the characterization with each progressive level, dose-response estimates also progress from more conservative (protective) to factually based (predictive) [32].

Mathematical dosimetry models that incorporate mechanistic determinants of disposition of chemicals have been useful in describing relationships between exposure concentration and target tissue dose, particularly as applied to describing these relationships for the dose-response component of risk assessment. The default dosimetric adjustments used in the RfC methods were based on comprehensive model structures and reduced to forms requiring a minimal number of parameters by utilizing the dominant determinants of disposition for various categories of compounds and simplifying assumptions [3,32].

For example, because a theoretical model of particle deposition requires detailed information on all of the influential parameters (e.g., respiratory rates, exact airflow patterns, complete measurement of the branching pattern of the respira-

tory tract) across the various species used in risk assessment, an empirical model (i.e., a system of equations fit to experimental data) was developed as the default model [3].

The default DAF calculated for gases, as for particles, is different for each respiratory tract region or for remote effects. In addition, the DAF for gases is dependent on which of 3 categories classifies the gas. The scheme used to categorize gases (Fig. 2) was constructed based on the physicochemical characteristics of water solubility and reactivity as major determinants of gas uptake. Reactivity is defined to include both the propensity for dissociation and the ability to react either spontaneously or via enzymatic reaction in the respiratory tract. The scheme does not apply to stable gases that exert their effects by reversible 'physical' interactions of gas molecules with biomolecules (e.g., 'displacement' of oxygen by carbon dioxide).

As an example, Fig. 3 shows the schematic for the default model used to characterize respiratory tract uptake of category 1 gases. Category 1 gases are defined as highly water soluble and rapidly reactive. Because of these properties, category 1 gases (e.g., hydrogen fluoride, chlorine, formaldehyde, and the organic esters) are likely to interact with the respiratory tract. The objective of the default modeling approach is to describe the effective dose to the 3 regions by addressing the absorption or 'scrubbing' of the gas from the inspired airstream as it travels from the ET to PU region. The approach used to model the uptake is based on the concept of an overall mass transfer coefficient, K_g [3,32]. The concept of the K_g is based on a concentration gradient analysis similar to Fick's Law of diffusion and is utilized to describe transport through several different phases, such as air and the liquid/tissue phase of the respiratory tract. A fractional penetration model is used to determine the fraction of the inhaled concentration in each region. For example, the uptake in the ET region and the output to the TB region (fractional penetration, fp_{ET}) is dependent on the K_{gET}, so that uptake in the ET region is defined as $1 - fp_{ET}$. A ventilation-perfusion model is used to estimate the uptake in the PU region by sub-

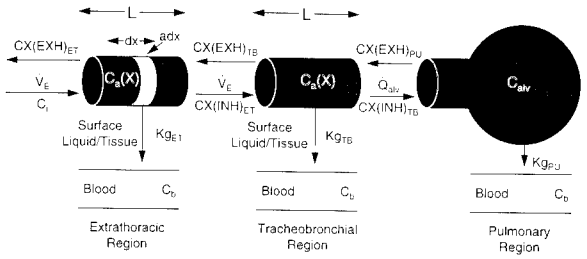


Fig. 3. Schematic of model to estimate default DAF_r for gases in category 1 [3,32]. a, airway perimeter; C_{alv}, pulmonary region gas concentration; C_b, blood concentration; C_i, inhaled concentration; $CX(EXH)_{ET}$, concentration existing from ET region upon exhalation; $CX(EXH)_{PU}$, concentration existing from PU region upon exhalation; $CX(EXH)_{TB}$, concentration exiting from TB region upon exhalation; $CX(INH)_{ET}$, concentration exiting from ET region upon inhalation; $CX(INH)_{TB}$, concentration exiting from TB region upon inhalation; dx, differential of axial distance into airway; ET, extrathoracic respiratory region; K_{gET}, overall mass transport coefficient of the ET region; K_{gPU}, overall mass transport coefficient of the PU region; K_{gTB}, overall mass transport coefficient of the TB region; alv, alveolar ventilation rate; $\dot{V}_E$, minute ventilation.

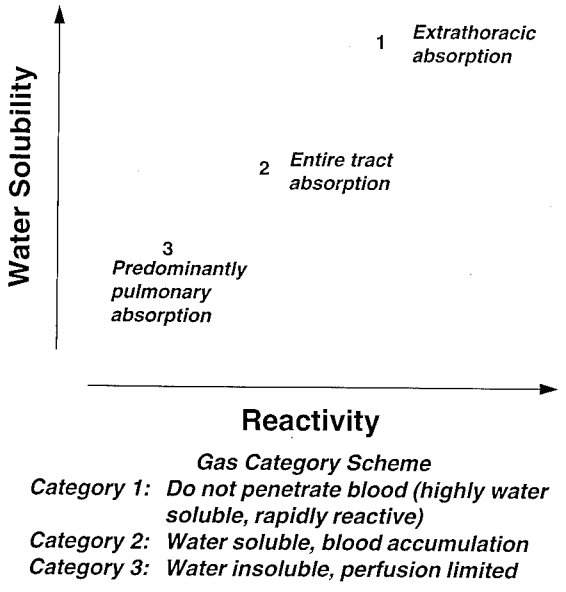


Fig. 2. Gas categorization scheme based on water solubility and reactivity as major determinants of gas uptake [3,32]. Reactivity is defined to include both the propensity for dissociation as well as the ability to serve as a substrate for metabolism in the respiratory tract. Definitive characteristics of each category and the anticipated location (region) for respiratory tract uptake are shown.

stituting the concentration of the air exiting the TB region for the inhaled concentration. The rate of mass absorbed at the gas-surface interface of the airway in a region (r) is simply the product of the absorbed fraction, $(1 - fp_r)$, and the total mass inhaled during a single breath, $\dot{V}_E C_i$, where C_i is the inhaled concentration. The $\dot{V}_E$ is used as the default volumetric flow rate because it approximates the flow rate at which the animal was breathing during the experimental exposure. The alveolar ventilation rate is used to calculate the absorption rate for the PU region.

The DAF_r for each region is then calculated based on equations describing the relationship between K_g and $1 - fp_r$ for each region, the ventilation rate, and regional surface area. The assumption that absorption is distributed equally within a region allows the description on a regional basis. Although this is a drastically reduced number of parameters in comparison to distributed parameter model descriptions, the default model does require K_g values for different animal species and gases. It is important to note that K_g is both species and chemical specific. Values of K_g obtained in a single animal species may be scaled within a species for a different gas in the same category by decomposing K_g to the individual gas-phase and surface-liquid/tissue phase transport resistances [3]. The default equations can be further reduced by applying additional simplifying assumptions regarding the likely values of K_g. The derivation of the equations and DAF_r for each region, including the models for the 2 other gas categories, are provided in detail elsewhere [3].

An understanding of the basis for the default adjustments allows development of a framework for the evaluation of whether an alternative model structure may be considered optimal relative to the default. Depending on the relative importance of these various determinants, models with less detail (i.e., less of the determinants depicted in Fig. 1) may be used to adequately describe difference in dosimetry for the purposes of interspecies extrapolation. An alternative model might be considered more appropriate than the default for extrapolation when default assumptions or parameters are replaced by more detailed, biologically motivated description or actual data, respectively. For example, a model could be preferable if it incorporates more chemical- or species-specific information or if it accounts for more mechanistic determinants. These considerations are summarized in Table 2. The sensitivity of the model to these differences in structure may be gauged by its relative importance in describing the response function for a given chemical.

5. Opportunities to replace default approaches with mechanistic data: vinyl acetate as an example

Improvements in the design of standard inhalation toxicity studies and development of adjunct mechanistic data should increase the accuracy of extrapolation and thereby reduce uncertainty. Given the default paradigm presented above, studies can be conducted to address

Table 2
Hierarchy of model structures for dosimetry and interspecies extrapolation

Optimal[a] model structure
Structure describes all significant mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
Uses chemical-specific and species-specific parameters
Dose metric described at level of detail commensurate to toxicity data
Default model structure
Limited or default description of mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
Uses categorical or default values for chemical and species parameters
Dose metric at generic level of detail

Source: Refs. 3 and 32.
[a] Optimal is defined as preferable or more appropriate relative to the default.

specific areas of uncertainty highlighted by the default approach and the range of UFs used in the default approach. Aside from using the RfC methodology as a guide in this process, all studies should be designed to answer specific questions; i.e., they should be hypothesis driven. Too often, toxicologists are resorting to standard study protocols with little thought given to the health question being addressed. Presented below is an example of the nasal toxicant vinyl acetate and how data on mode of action can be used to address many of the default factors used in the conventional RfC methodology.

5.1. UF: duration adjustment and choice of dosimetrics

As discussed above, the underlying assumption of Eq. 2, that the resultant continuous human equivalent exposure concentration should be the $C \times t$ product equivalent of the experimental animal exposure, is essentially 'Haber's Law'. Accordingly, a constant, in this case a fixed effect level (i.e., a constant severity and/or incidence level) is assumed to be related to the $C \times t$ product. Toxicity, however, can depend on the magnitude, duration, and frequency of exposure. If detoxication or elimination occurs between successive doses, then the $C \times t$ product may not be an appropriate dose metric and the duration adjustment becomes too conservative. The choice of an appropriate measure of dose must be defined by the nature of the pathogenesis process for the effect under consideration. Concentration and time study designs may provide insight on whether concentration, duration, or the product most influence the toxicity. Because dosimetry models can integrate time- and concentration-dependent processes (e.g., distribution and metabolism rates) that are important mechanistic determinants of toxicity, they can eliminate the need for the default $C \times t$ product duration adjustment and provide insight on the proper dose metric to use [35]. These models can be linked to pharmacodynamic models that address factors influencing tissue response and further refine the choice of dose metric. In the case of vinyl acetate, it is the acetic acid produced within sustentacular cells of olfactory epithelium that initiates cytotoxicity. Cell death is likely a consequence of heavy tissue proton burdens that overwhelm the natural cellular buffering and proton transport mechanisms [36,37]. Such a mechanism suggests that response is dependent on the intracellular proton concentrations as a dose metric. PBPK models have the capability to account for many of these factors in a chemical- and species-specific manner (Table 3). A recent review of these methods for nasal toxicants illustrated a variety of approaches to using dosimetry models to address these issues [38]. A model of vinyl acetate deposition and metabolism has recently been developed [37] (Fig. 4). The model construct is similar to that developed by Morris et al. [39]. Dosimetrics for acetic acid and acetaldehyde formation are generated for specific sites within the nose such as respiratory vs. olfactory epithelium. A human-equivalent model can be developed in which human-specific data on airflow characteristics [40] and metabolism [41] are incorporated into the model. These attributes of the model enable a more close mechanistic link between the specific tissue affected and the tissue dosimetry of the toxic metabolite causing olfactory degeneration (acetic acid). This is an improvement over

Table 3
Advantages of PBPK/dosimetry modeling approach

- Allows integration and extrapolation using diverse data
- Predicts complex kinetic behavior
- Capability to 'lump' or 'split' model structure to explore dose response
- Enables interspecies dosimetric comparisons
- Allows parameter scaling across species
- Facilitates hypothesis generation
- Identifies areas of needed research

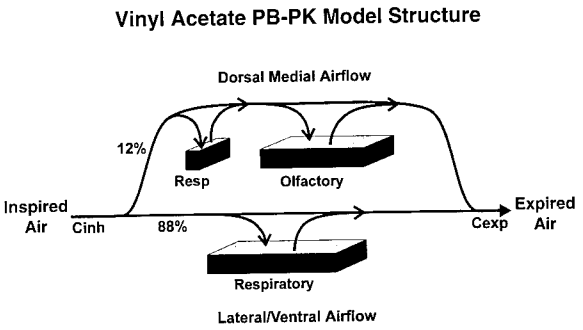


Fig. 4. Schematic diagram of the vinyl acetate PBPK model. The model structure, developed by Morris [39] is based on airflow patterns and anatomic, physiologic and biochemical data specific to the nasal cavity.

the default RfC dosimetry approach in a number of ways including providing site-specific information on metabolism (as opposed to a regional K_g). The use of this model should therefore result in a reduction in the residual UF of 3.

5.2. UF: completeness of database

An incomplete data set will frequently result in the application of a 10-fold UF. Vinyl acetate has been tested in 90-day and 2-year inhalation studies in both rats and mice in which the pathology of URT lesions has been characterized by extensive histopathology examination. A study design using adequate numbers of animals and interim sacrifices, ensured reasonable statistical power. These studies showed the critical effect is degeneration of the olfactory epithelium with NOAEL of 50 ppm. A significant incidence of nasal tumors was observed at the highest concentration of 600 ppm. Occupational exposure data show no adverse effects of exposure at mean exposure levels of approximately 8.6 ppm. Inhalation developmental toxicity studies showed there was no embryolethality or developmental toxicity at levels below maternally toxic levels. Although an inhalation reproductive toxicity study has not been conducted, a multigeneration drinking water study was negative. Thus, with the possible exception of an inhalation reproductive toxicity study, this database was considered complete. However, the lack of evidence for systemic toxicity, coupled with estimates of nasal metabolism that suggest complete metabolism within the nose and dosimetry data indicating insignificant remote distribution of vinyl acetate (discussed below), obviate the need for studies of reproductive toxicity. Thus, there is no need for an UF to account for these data gaps.

5.3. UF: subchronic to chronic

In the case of vinyl acetate, there is no need for the application of an UF to account for the lack of a chronic inhalation bioassay. However for many chemicals such data will not be available and will require the uncertainty of unknown outcomes from chronic exposure to be addressed. Adjunct data on metabolism that bear on the question of accumulation of toxic metabolites and lesion repair rates could be important factors for reducing this uncertainty. In cases where toxic metabolites or lesions do not accumulate over time one might expect that toxic levels of exposure would be similar despite the length of exposure. This is the case for many irritants. Lesion repair often begins during exposure and may not be observable at time of sacrifice. As an example, recovery of olfactory epithelial damage was observed to commence prior to cessation of methyl bromide exposures [42]. Incorporation of interim sacrifices into standard inhalation bioassay study designs could provide information on the chronological dynamics of lesion formation and repair. In an empirical approach to the issue of differences in toxic exposure levels between subchronic and chronic exposures and the appropriateness of UFs, Nessel et al. [43] summarized the $LOAEL_{subchronic}/LOAEL_{chronic}$ for 9 studies, the majority of which showed portal-of-entry effects. The mean ratio was 4.5 suggesting that a factor 10 might be excessive for these types of chemical.

5.4. UF: LOAEL to NOAEL

Since inhalation studies are difficult to conduct, the toxicologist is frequently faced with less

than adequate range finding data to support the choice of exposure concentrations for the definitive bioassay. In addition, maintaining accurate exposure levels can be technically difficult resulting in excursions above or below the target concentration. These factors, among others, can contribute to a target NOAEL becoming a LOAEL. Information which could help reduce this uncertainty includes structure activity relationships showing NOAELs for similar compounds. The benchmark dose approach offers an advantage in this situation since the model allows the estimation of NOAELs. Toxicologists designing inhalation bioassays should be knowledgeable about this approach in order to gain the complete advantages of its implementation. The approach rewards statistically robust study designs (large numbers of experimental groups, large numbers of animals per group) with better estimates of the NOAEL and lower bound estimates that are close to the maximum likelihood estimates. The cost of the improved study design must be weighed against its potential benefit. In the case of vinyl acetate, NOAELs were determined in all studies. However, because the benchmark dose approach allows interpolation between exposure levels, the benchmark estimate turned out to be higher than the lowest concentration tested (50 ppm). Therefore no UF is necessary.

5.5. UF: laboratory animal to human and intrahuman variability

As explained above, what is known about interspecies differences in respiratory tract dosimetry ('pharmacokinetic' processes) and tissue response ('pharmacodynamic' processes) suggest that these differences are significant. As noted in Table 1, pharmacokinetic and pharmacodynamic processes fall under the purview of both the interspecies and the intrahuman UFs and the factors are typically parcelled into factors of 3 each. These factors are shown incorporated into the exposure-dose-response framework in Fig. 5. The default UF for interspecies extrapolation is $10^{0.5}$ or approximately 'halved' in the RfC methods because application of the default DAF_r

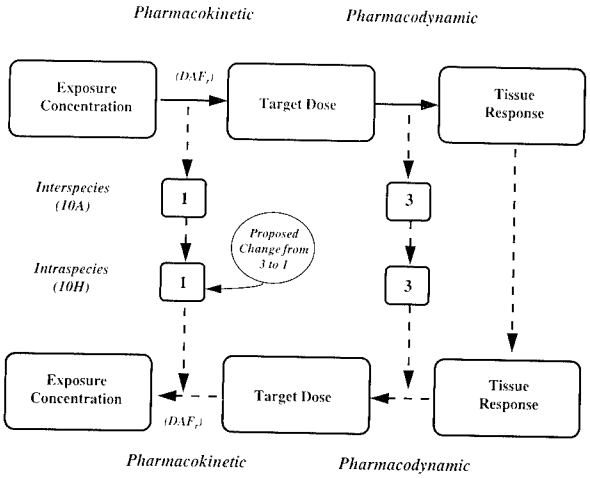


Fig. 5. Interspecies and intraspecies (intrahuman) UFs incorporated into framework of exposure-dose-response continuum (Ref. 45, as modified from [46]).

was thought to account for a portion of the 'pharmacokinetic' portion of the extrapolation. Although the default DAF_r also adjusts for factors that control target dose in humans, a portion of the uncertainty was left remaining because the adjustments are viewed as default. More robust dosimetry models can be anticipated to obviate the entire pharmacokinetic component. In the case of vinyl acetate, the proposed PBPK model [37] can be scaled to humans and used with human-specific parameter values. Use of these robust dosimetry models to simulate the experimental animal and anticipated human exposure scenario in order to calculate the HEC estimates could obviate both parcels of the pharmacokinetic component.

Models that address the determinants of response may impact the pharmacodynamic component. In the case of the URT, few diseases are known to be pre-existent and not attributable to some source of ambient exposure. Effects such as rhinitis, rhinorrhea, irritation, and swelling of the mucous membranes are shared among those caused by airborne allergens and low-level chemical exposure [44]. Thus, it is possible that atopic status or pre-existing URT disease may represent susceptibility factors for chemical exposures that cause URT damage.

With regard to the mechanism of vinyl acetate

toxicity, populations with reduced olfactory capacity and those with high nasal carboxylesterase activities would be expected to show sensitivity. Studies on human nasal carboxylesterase with vinyl acetate showed a relatively tight range of activities for both respiratory and olfactory epithelium [41]. The sample population was from 9 donors and included predominantly caucasian male donors aged 54–82 years. To the extent that there is some homogeneity within this population, an argument could be made that some reduction in the pharmacodynamic portion of the UF is warranted since the rates of metabolism used in the model reflected this variability.

6. Conclusions

This overview illustrates the many complexities involved with assessing risk of inhaled materials and highlights the many assumptions that contribute to uncertainty in these assessments. However, by conducting toxicological research in a manner that is aimed at addressing uncertainties, more meaningful and realistic assessments can be made. Physiologically based approaches to modeling dosimetry of inhaled materials is becoming more common. Basic research on mechanisms of action is needed to enable development of response models. With regard to both dosimetry and response, research on human-specific parameters appears to be the area in greatest need for research.

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Pyrethroids, nerve poisons: how their risks to human health should be assessed

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Abstract

The extensive worldwide efforts of structural modification of natural pyrethrins for better performances have resulted in successful development of a wide variety of synthetic pyrethroids with tremendously high efficacy, knock-down activity or vapor action, and/or with acceptable environmental stability and safety. Currently these pyrethroids including their preferentially manufactured stereoisomers are widely used in agriculture, and for public health as well as household insect control. The detailed toxicology and metabolism studies intended to attain human risk assessment have revealed that with voltage-dependent sodium channel as target site pyrethroids induce pronounced repetitive activity characterized grossly by tremor, hypersensitivity, choleoathetosis, and salivation. In addition, so-called cyano-pyrethroids cause transient skin paresthesia in workers. With regard to tumorigenicity, mutagenicity, teratogenicity and developmental toxicity, no significant findings have been reported. Pyrethroids are eliminated from the animals quite rapidly and completely, undergoing oxidation and ester hydrolysis followed by various conjugations, with low tissue residues. Thus, overall, sound scientific bases exist for human risk assessment under the present usage conditions.

Keywords: Pyrethroids; Risk assessment; Skin paresthesia; Esterase; Sodium channel

The pyrethroid constitutes one unique group of insecticides which possess ample possibility of structural modification, to attain diversified characteristic features. Namely, immediately after elucidation of chemical structure of 6 insecticidal entities in chrysanthemum flowers just after World War II (Fig. 1 depicts pyrethrin-I, one of the major components), extensive efforts were made to modify both acid and alcohol moieties, to lead to synthetic pyrethroids with a variety of chemical structures.

No one had ever thought that such a diversified degree of modification, which has been successfully attained in structure as well as in biological activity, could have resulted in opening a new era in our pest control strategy with pyrethroids. Fig. 2 shows 5 examples of such a

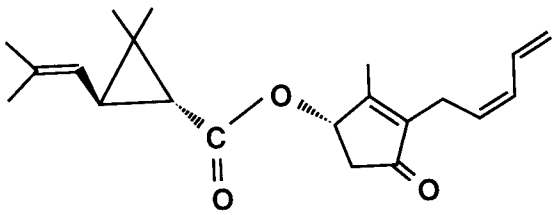


Fig. 1. Prethrin-I.

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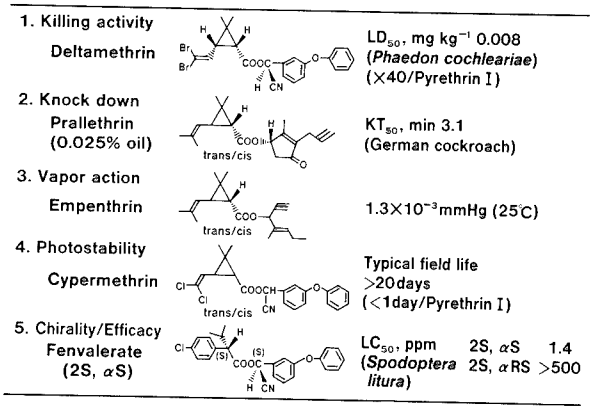


Fig. 2. Pyrethroids with different characteristics.

variety of activities of pyrethroids including a remarkable high killing activity with deltamethrin, rapid knock down activity by prallethrin, vapor action at room temperature by empenthrin, and photostability of cypermethrin as well as deltamethrin and fenvalerate. In many of these respects synthetic pyrethroids surpass natural pyrethrins. One of the characteristics of pyrethroids is a tremendous difference in biological activity among chiral isomers, fenvalerate being one such example; its [2*S*,α*S*] isomer is approximately 500 times more active than the [2*R*,α*RS*] counterpart, and specific chiral isomers such as deltamethrin and esfenvalerate or fenvalerate isomer are commercially prepared and being marketed.

Thus, depending on these characteristic properties, some pyrethroids are being used worldwide for household insect control and public health purposes, whereas others are extensively used in agriculture owing to the increased photostability, yet with the acceptable environmental safety coupled with the desired efficacy. With respect to acute toxicity in mammals, generally pyrethroids are of low toxicity, certain members having LD_{50} values of above 1000 mg/kg body wt. Moreover, pyrethroids show a much higher selective toxicity ratio, implying they can be used more safely [1]. In subsequent parts of the paper the present status of mammalian toxicology of pyrethroids is briefly dealt with which will give some thoughts on the role of toxicology for tomorrow's risk assessment of pyrethroids.

As neuropoisons, pyrethroids develop a variety of toxic symptoms in mammals, as exemplified in Table 1. Traditionally, these symptoms are classified into Type I and Type II [2]: Type I symptoms, mainly observed in so-called non-cyano compounds, are characterized by tremor, aggressive sparring and increased sensitivity to external stimuli, whereas cyano-pyrethroids develop mainly salivation and choreoathetotic movement abbreviated as CS-syndrome. However, some pyrethroids including fenpropathrin and cyphenothrin show mixed T and CS symptoms. Neurotoxicity studies in animals reveal decreased motor activity and modified acoustic startle response in amplitude and latent periods, although no data are shown here.

Table 1
Toxic symptoms (Type I and Type II) of pyrethroids in mammals

Type	Pyrethroids	Symptoms
I	Pyrethrin	*T-syndrome*
	Allethrin	Aggressive sparring
	Tetramethrin	Increased sensitivity to external stimuli
	Resmethrin	Prostration
	Phenothrin	Tremor
	Permethrin (non-cyano)	
II	Cypermethrin	*CS-syndrome*
	Deltamethrin	Pawing and burrowing
	Fenvalerate	Salivation
	Cyhalothrin (cyano)	Clonic seizure
		Choleoathetosis
Mixed	Fenpropathrin	*T-syndrome/CS-syndrome*
	Cyphenothrin (cyano)	

The toxic symptoms of pyrethroids observed in humans are considered similar to a certain extent, to those in mammals, although no reliable reports on death by professional exposure have been available. Two additional points should be mentioned here:

(1) these symptoms are observed mainly in applicators of cypermethrin, deltamethrin, or fenvalerate, all so-called cyano-pyrethroids, used for agricultural purposes. Non-cyano pyrethroids used for household insect control such as allethrin, tetramethrin and phenothrin have no such episodes of intoxication probably due in part to low exposure in humans;

(2) skin paresthesia or facial sensation developed in humans will be dealt with in detail later.

The pyrethroid has been demonstrated up to now not to act on any known enzymes. Experimentally, the principal action of pyrethroids in nervous systems is to induce pronounced repetitive activity. The intensity of repetitive discharge due to depolarizing after potential varies greatly with pyrethroid structure; generally, the cyano-pyrethroids cause long-lasting trains [3]. In addition, the cyano-pyrethroids develop continued depolarization of resting membrane, which leads to conduction block.

As is well known, the basis for such disturbance of nerve impulse by pyrethroids lies in their action onto the voltage-dependent sodium channel of excitatory nerves, to alter permeability of the sodium ion. Actually, the pyrethroid is known to give rise to prolongation of sodium current, as evidenced by so-called patch clamp method, where sodium current is recorded under artificial depolarization and repolarization. After termination of depolarization the control sodium current rapidly declines to zero level (dashed line in Fig. 3), whereas sodium tail current remains after exposure to pyrethroids [4]. This so-called tail sodium current results from slow closing of the sodium channel. The altered sodium current caused by Type II pyrethroids, here cypermethrin, lasts longer, as compared with Type I pyrethroids such as permethrin.

The primary amino acid sequence of the α-subunit of the sodium channel has been determined [5] and 5 distinct neurotoxin binding sites are established. However, the pyrethroid as well as dichlorodiphenyltrichloroethane (DDT) is presumed not to have affinity to any of these binding sites [6], and thus a 6th binding site is occasionally postulated.

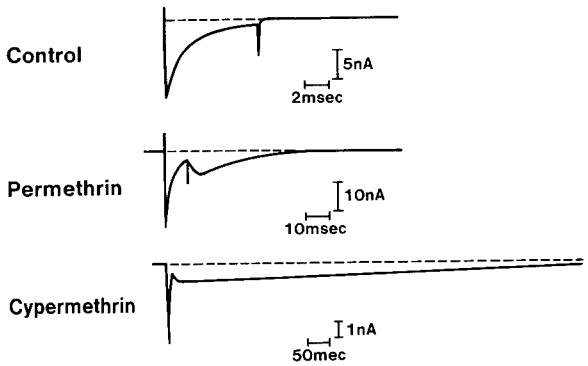


Fig. 3. Effect of pyrethroids on sodium currents in myelinated nerve fibers of *Xenopus laevis*.

Generally, depolarization of nerve membrane by Type I and Type II pyrethroids leads to release of transmitters such as acetylcholine, dopamine, norepinephrine and glutamic acid [7–9]. In addition, Type II pyrethroids reportedly bind to the gamma-aminobutyric acid (GABA) receptor [10], or inhibit action of calcineurin [11]. These respectively would result in transmitter release as well, and thereby develop a variety of toxic symptoms. Free cyanide does not appear to be involved in the toxicity manifestation [12]. Thus overall successive underlying events are apparently more complicated in Type II pyrethroids than in Type I compounds, as shown in Fig. 4, although no detailed molecular mechanisms are yet to be revealed.

As antidotal agents against intoxication by pyrethroids, skeletal muscle relaxants such as mephenesin and methocarbamol prove effective [13,14]. Atropine efficaciously suppresses salivation by Type II pyrethroids without, however, any effects on survival of intoxicated animals [15]. Anticonvulsants like barbiturates and diazepam are not so effective [16,17].

With a wide spread usage of pyrethroids, typically photostable, cyano-containing com-

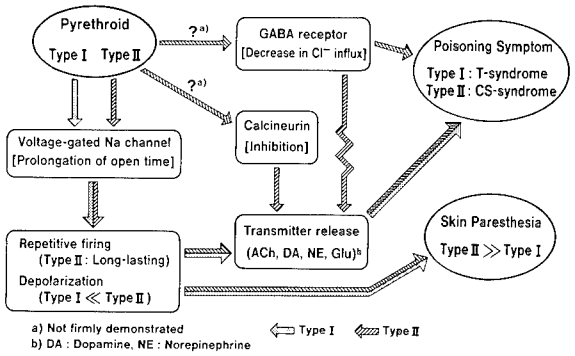


Fig. 4. Hypothetical sequence of pyrethroid-induced poisoning symptoms.

pounds, workers exposed to them have experienced so-called skin paresthesia or facial sensation which is characterized by transient burning/tingling/itching sensation of the exposed skin. An experimental model for the symptoms has been established, where rabbits are treated with pyrethroids on the back, and frequency of licking/biting of application site is grossly observed (Suzuki et al., unpublished).

The intensity of skin paresthesia by a number of pyrethroids has been tested by using the animal model, and the results are summarized in Fig. 5. Consistent with human observations, the cyano-containing pyrethroids develop severe symptoms of skin paresthesia. Other non-cyano compounds beginning with permethrin, and natural pyrethrins, also possess such an activity,

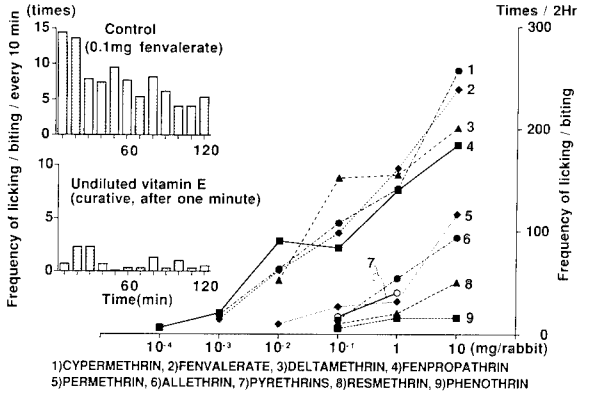


Fig. 5. Skin paresthesia by pyrethroids in rabbits and therapeutic effects of vitamin E.

although quite weak, which, usually not widely recognized, has been occasionally reported for certain human populations in the past. Several materials including vitamin E are found to effectively mitigate the characteristic response of rabbits, as seen on the left hand side of Fig. 5, which is actually comparable to the negative control. The findings again agree with human observations.

Thus, overall, the characteristics of skin paresthesia are summarized here: cyano-pyrethroid >> non-cyano pyrethroid; transient burning/tingling/itching sensation of skin; limited in exposed area; from 0.5–1 h to several hours after exposure; light, heating, wind – aggravation factor; no inflammatory reaction (no erythema, no edema); no functional or histopathological changes; protective clothing – effective; effective preventive agents – vitamin E, mineral oil, cream containing vitamins A and D, benzocaine.

The skin paresthesia is thus different from usual skin irritation or skin sensitization. Not only vitamin E, but also mineral oil, facial cream preparation containing vitamin A and D, and benzocaine are effective in preventing onset of the symptoms, whereas diphenhydramine, indomethacin or zinc oxide paste are without any mitigating effects.

The skin paresthesia is in a sense a unique toxic symptom, and at present no detailed molecular mechanism has been proposed, although repetitive firing in peripheral skin nerves due to pyrethroids has been postulated. Likewise no plausible mechanism of action of preventive agents has been proposed. In this regard, Narahashi has indicated that vitamin E or α-tocopherol possesses suppressing effect of tetramethrin-induced sodium current changes in rat cerebellar Purkinje cells, suggesting the action of α-tocopherol at sodium channel (Narahashi, unpublished). This finding may throw insight into the molecular mechanism of skin paresthesia observed particularly with cyano-containing pyrethroids.

Although no particular mention is made of other features of pyrethroid toxicity, including tumorigenicity, mutagenicity, teratogenicity and developmental toxicity, no significant findings

exist that may affect risk assessment to human health, so far as available information in open literature is concerned. With regards to metabolic degradation in mammals, to be closely related with the risk assessment the general tendency is that pyrethroids are eliminated from the animal body quite rapidly and substantially completely, as shown on the left side of Fig. 6 [18]. As shown on the right side, pyrethroids undergo extensive metabolism by means of oxidation and hydrolysis, followed by several conjugation reactions [12,19–21]. Depending on chemical structure the respective pyrethroids preferentially undergo oxidation and hydrolysis [20]. The pyrethroid esterase(s) in the mammalian liver that are localized mainly in the endoplasmic reticulum are apparently identical with esterases acting on malathion and *p*-nitrophenyl acetate [11].

Tissue residues of pyrethroids are generally quite low [18], and although the parent pyrethroids tend to be very lipophilic, sometimes similar to DDT, no bioaccumulation is observed after subacute dosing to mammals (Kaneko et al., unpublished). As seen on the right hand side of Fig. 7, the radiocarbons derived from fenvalerate disappear quite rapidly from organs and tissues, as compared with DDT. The high ^{14}C content in fenvalerate-treated rat ovary is actually due to lipophilic acid metabolite to be mentioned below. The ^{14}C in fenvalerate-treated animals is mainly the parent compound, while DDT is the sum of DDT, DDE and dichlorodiphenyldichloroethane (DDD). To the knowledge of present authors, these general

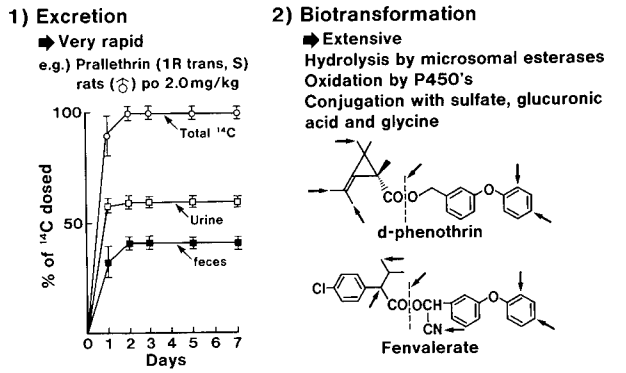


Fig. 6. General features of pyrethroid metabolism-1.

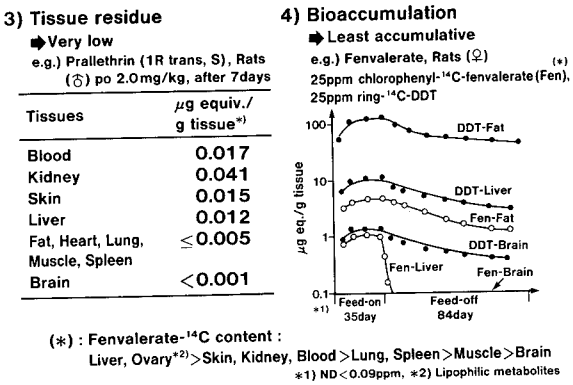


Fig. 7. General features of pyrethroid metabolism-2.

features of metabolism can be applied to most of the pyrethroids tested so far.

To have understanding of metabolic activity in humans, several human cytochrome P450 (P450) genes have been expressed in yeast through conventional gene engineering techniques [22], and the respective P450 isozymes are tested in vitro with regard to their preferential activity onto pyrethroids. The preliminary results are shown in Fig. 8 (Kaneko et al., unpublished). It appears that a limited member of P450s are responsible for oxidation of specific sites in the pyrethroid molecule. This information should be compared with the activity of resolved P450 enzymes in rats and other mammals, to gain a

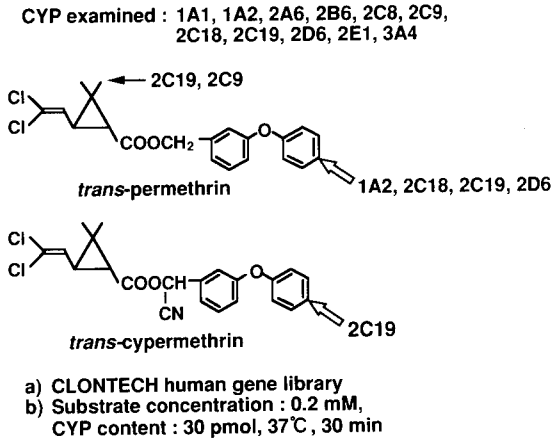


Fig. 8. Metabolism of pyrethroids by human P450 expressed in yeast.

better understanding of overall views of xenobiotic metabolism in animals and man.

In order to correlate age-dependent, differential toxicity of pyrethroids with metabolic capability of baby animals, ester hydrolysis as well as P450-mediated oxidation of pyrethroids is tested in vitro with liver microsomes from new-born rats (Kaneko et al., unpublished). As shown in Fig. 9, the activity of both enzymes is significantly low just after birth, namely 10% for esterase and 40% for mfo (mixed function oxygenase) 3 days after birth, as compared with the activities in 8-week-old animals more or less conventionally used for routine metabolic studies. It appears that this lower degradation activity in new-borns is at least one factor in higher susceptibility to pyrethroid toxicity.

One specific example where metabolism is closely associated with manifestation of pyrethroid toxicity can been seen in fenvalerate-induced granulomatous changes in animals. When fenvalerate is administered subacutely to mammals, histopathological changes develop in certain organs and tissues, including liver, spleen and lymph nodes, diagnosed to be granulomatous changes resulting from aggregation of macrophages and multi-nucleated giant cell infiltration [23]. Among 4 chiral isomers, only so-called Bα isomer, more exactly [2*R*,α*S*] isomer (Fig. 10) has been demonstrated to develop the tissue changes [23].

In search of the mechanism of granuloma formation by fenvalerate Bα, extensive biochemical and toxicological studies have been conducted [24]. As shown in Fig. 10, only fenvalerate Bα produced the specific metabolite in mammals, which is identified to be 2*R*-CPIA-cholesterol ester. This novel cholesterol ester is formed by cholesterolysis of fenvalerate Bα ester bond by a microsomal esterase activity, and this cholesterol ester proves to be the causative agent of granulomatous changes. The enzyme responsible for this novel reaction appears to be a microsomal esterase, with microsomal cholesterol as acceptor of intermediary acyl moiety of fenvalerate Bα. Roughly, in parallel with CPIA-cholesterol formation, mammals develop granulomatous changes.

Based on all the available information presented here, fairly extensive toxicology studies have been conducted with pyrethroids, and sound scientific bases exist for the risk assessment under present usage conditions. However, a couple of points should be referred to for future research needs of pyrethroid toxicology and better risk assessment:

(1) determination of exact molecular targets on sodium channel;

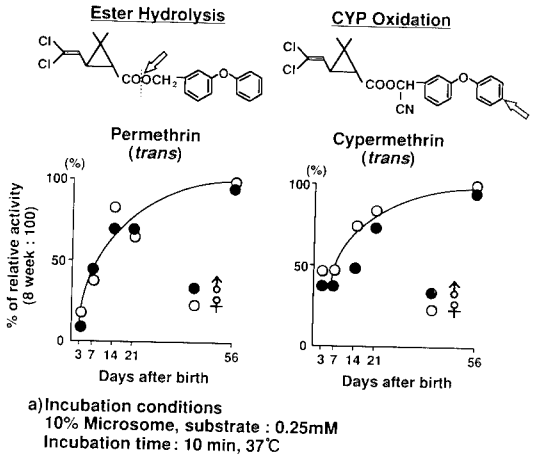
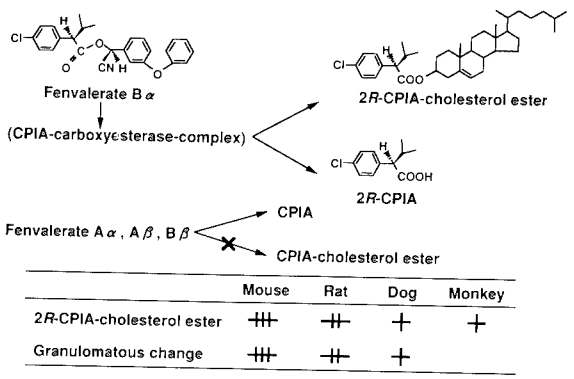


Fig. 9. Age-dependent metabolism of pyrethroids in vitro by rat liver microsomes.



	Mouse	Rat	Dog	Monkey
2*R*-CPIA-cholesterol ester	+++	++	+	+
Granulomatous change	+++	++	+	

Fig. 10. CPIA-cholesterol ester as causative agent of granulomatous change.

(2) elucidation of role of pyrethroid-dependent alteration in nerve transmission, including (i) molecular basis of Type I and Type II symptoms and (ii) exact mechanism of skin paresthesia;
(3) elucidation of species- and age-dependent features of toxicity and metabolism;
(4) as usual the minute modification of chemical structure of respective pyrethroid compounds including geometry and chirality sometimes profoundly affects toxicity and metabolic profiles. Therefore, similarity and difference among a variety of pyrethroid compounds should be pursued further so as to integrate structure-activity relationship in toxicity and metabolism before it can be stated as a general term that the pyrethroid possesses such and such features.

Thus, these researches, when accomplished, will certainly contribute to refining hazard identification, dose-response extrapolation and exposure analysis, and thereby contribute to facilitating rational approach to risk characterization.

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Toxicology Letters

Ferritin as a source of iron and protection from iron-induced toxicities

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Abstract

The iron storage protein ferritin can contribute to or protect against toxicities which involve iron. Iron can catalyze the oxidation of lipid, protein, DNA and various biomolecules that can reduce iron. Iron can be reduced and released from ferritin by the free radical form of various toxins or superoxide resulting from oxygen reduction by chemicals which redox cycle. Iron can also increase ferritin synthesis by an iron-binding protein which releases from an iron-responsive element in mRNA for ferritin. This increase in ferritin synthesis provides a non-reactive storage site for iron. The mechanism by which iron is placed into ferritin is unknown. We propose that it is catalyzed by ceruloplasmin, the copper-containing ferroxidase that loads iron into transferrin. We believe that the ferroxidase activity, thought to reside in the heavy chain of ferritin, is an artifact resulting from ferrous iron autoxidation. We load iron into ferritin with ceruloplasmin so ferritin plus ceruloplasmin is an effective 'antioxidant'.

Keywords: Iron; Ferritin; Ceruloplasmin; Oxidative stress; Lipid peroxidation

Ferritin is a widely distributed iron-storage protein thought to be very important to provide protection against the catalysis of deleterious oxidation of biomolecules by iron [1]. Ferritin can store up to about 2500 atoms of iron in a ferric oxyhydroxide core surrounded by phosphate [2]. The core is surrounded by 24 subunits of heavy and light chain proteins [1]. Ferritin may also serve as a source of iron for the synthesis of iron-containing enzymes when iron absorption is insufficient [3].

Most investigators believe that ferritin has its own ferroxidase activity such that its incubation with ferrous iron results in incorporation of the iron into the ferric iron core [4]. We do not subscribe to this mechanism as the ferroxidase activity of ferritin is dependent upon the use of a buffer which promotes the autoxidation of iron and the iron association with the ferritin is in a form that is not as stable as is the iron in isolated ferritin [5]. In addition, the loading of ferrous iron into ferritin using its 'ferroxidase' activity results in the oxidation of the protein [5].

We use the ferroxidase activity of ceruloplasmin to load iron into ferritin [5,6]. Ferritin loaded with ceruloplasmin behaves very similarly to ferritin isolated from tissues, both with respect to the maximum amount of iron that can be loaded or found in ferritin and the properties of the iron incorporated into or in native, isolated ferritin [5]. Ceruloplasmin is generally considered a serum protein but we [7] and others [8]

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have shown that the mRNA for ceruloplasmin indeed does exist in cells. However, there is no hard evidence that tissue ceruloplasmin is the enzyme responsible for loading iron into ferritin in tissues. We can only gather evidence to support the hypothesis. We have shown that the iron in ferritin loaded by ceruloplasmin behaves similarly to iron in ferritin isolated from tissues, that the rate of iron loading into ferritin is maximum at a 1:1 molar ratio of ceruloplasmin to ferritin, that ceruloplasmin forms a complex with ferritin, and that the maximum amount of iron loaded into ferritin (~2500 atoms) is the same as the total amount of iron that can be found in ferritin in vivo.

The proposal that ceruloplasmin may be the ferroxidase that loads iron into ferritin is also attractive by the fact that the oxidation of iron by ceruloplasmin results in the reduction of molecular oxygen completely to water without the release of partially reduced species of oxygen [9].

The oxidation of DNA, protein and lipid can be promoted by the hydroxyl radical generated by Fenton reagent (H_2O_2 and ferrous iron), or by as yet undescribed mechanisms [10,11]. The latter is probably best exemplified by the oxidation (termed peroxidation) of lipid (polyunsaturated fatty acids) where one proposal is that the hydroxyl radical is involved, whereas others believe that a 1:1 ratio of ferrous to ferric iron causes lipid peroxidation [10,11]. Superoxide, resulting from toxins that can redox cycle, such as by paraquat [12,13], adriamycin [14], etc., can promote lipid peroxidation by a superoxide-driven Haber Weiss sequence of reactions where the superoxide serves as a source of reducing equivalents to reduce iron and dismutating to provide H_2O_2 for Fenton reaction.

Ferritin may be of interest in toxicities or pathologies involving iron for 2 reasons and with 2 opposite roles. Ferritin could provide a source of iron or it could be protective, by sequestering the iron in a non-reactive state. We showed that lipid peroxidation can occur during oxidation of ferrous iron by ceruloplasmin [15]. The addition of ferritin inhibited this lipid peroxidation, presumably by providing a 'safe' place for sequestration of the ferric iron.

Superoxide resulting from redox cycling toxins and the free radical form of many toxins are able to reduce iron in ferritin [16–18]. Organic radicals are much more efficient than superoxide in reducing iron in ferritin [18], however they also react very rapidly with molecular oxygen to produce superoxide, perhaps somewhat limiting the rate of release of iron from ferritin during redox cycling.

Iron can also directly oxidize some biomolecules, such as some sulfhydryl compounds, biogenic amines and ascorbic acid. (The reactions can be stated the other way, that these biomolecules are good iron reductants.) Sometimes these chemicals are therefore said to autoxidize to form oxygen radicals which can contribute to toxicities. This subject will be addressed in more detail below. In the extreme, one of these chemicals, ascorbic acid, is often used to promote oxidations. Ascorbic acid and iron are often used for a model lipid peroxidation system [19]. The ratio of ascorbic acid to iron is important, as is the chelator used for the iron if it is something other than the ascorbic acid [20]. Ascorbic acid will promote lipid peroxidation when the concentration is insufficient to keep all of the iron reduced. The antioxidant activity of ascorbic acid can be observed when the ratio of ascorbic acid to iron is sufficient to keep all of the iron reduced because it eliminates the required ferric iron. This is undoubtedly related to the physiological situation. Sufficient ascorbic acid is in some tissues to keep the small amount of available iron all reduced, presumably until it can be placed into ferritin. Ascorbic acid obviously does not usually promote lipid peroxidation in vivo. However, it may in cases of tissue injury where the concentration of iron becomes too high to be completely reduced by the ascorbic acid. It is also unreasonable to propose that ascorbic acid, or similar biomolecules, can release iron from ferritin. Such would be extremely deleterious. Ascorbic acid reduces contaminating iron, which can autoxidize to produce superoxide. Thus, ascorbic acid will release iron from ferritin by reduction of the iron, using superoxide, when contaminating iron gets reduced by ascorbic acid to start this obviously non-physiological system.

This presumably does not occur in the physiological situation or ascorbic acid would be toxic. This could, however, be the case in trauma or other cases of tissue injury.

A host of other biomolecules and other chemicals have been reported to be toxic by a prooxidant effect, usually proposed to involve oxygen radicals. These include a variety of thiols [21], biogenic amine, etc. However, such is probably not the case except in certain pathological situations or with model in vitro systems. For examples, various biogenic amines, thiols and other iron reductants have been used in in vitro toxicity studies, frequently involving cells in culture. Unfortunately the actual active chemical is probably a transition metal but they are frequently not studied or even mentioned in these in vitro studies. Only in a few cases have investigators shown that desferrioxamine, an effective stable iron chelator, will block the deleterious effect of the chemicals being studied.

Frequently these difficulties arise as a result of the fact that many of these chemicals are also excellent iron chelators. The commercial preparations of many biomolecules are contaminated with iron. In other cases, the iron is a contaminant in water or buffers. Sometimes considerable effort must be taken to remove this iron. For example, it is necessary to incubate epinephrine with desferrioxamine for 72 h in order to eliminate the iron-catalyzed 'autoxidation' of epinephrine [22].

Iron does seem to be involved in various toxicities [23]. In these cases iron must be released from ferritin, iron-sulfur proteins or heme proteins. We know that iron can be released from ferritin by certain redox active toxins [18] and by superoxide [24]. The possibility that ferritin may serve as a source of iron in various toxicities has been reviewed by Reif [25]. Upon release from ferritin, the iron would be in the reduced form. Thus it is necessary to consider the oxidation of this iron if it were to be involved in toxicity. Then the rate of subsequent reduction must be considered. Toxicity might be limited if reduction does not occur or, conversely, if it does occur, the rate must be sufficient enough to keep all of the iron reduced. The biological reductant for this role has not been identified and neither has the iron chelator. Some chelators which have been proposed, such as ADP, citrate, etc., do not seem logical, for iron chelated with these chemicals redox-cycle rather rapidly and would therefore probably be toxic. They might therefore be involved in certain pathologies or toxicities but it is very unlikely that they would chelate iron in the normal situation. Likewise, it is difficult to identify the physiological reductant. It may be ascorbic acid, however, this seems unlikely as ascorbic acid is not a very strong iron chelator. And it is not glutathione. Glutathione is a very poor iron reductant [21] probably because it is not an iron chelator. And it is highly unlikely that other thiols, such as dihydrolipoic acid, are physiological iron reductants or antioxidants as they do not exist in the free form at very high levels. High levels would be required for dihydrolipoic acid to be an antioxidant as it is not a good reductant of iron.

It is thus apparent that many reactions and other factors must be considered when appraising the role of iron in catalyzing the oxidation of biomolecules. The 2 basic reactions are iron reduction and oxidation but both may occur with various reductants and various oxidants, some of which have more potential toxicological relevance than others but in all cases the reaction rates are dependent upon the iron chelators. The first reaction to study is the reduction of iron by physiologically relevant reductants (i.e., glutathione or ascorbic acid). The second reaction is iron oxidation, probably most importantly by molecular oxygen but also by H_2O_2 (the concentration of molecular oxygen, limited due to its relatively low solubility, is low, ~240 μM, but much higher than the concentration of H_2O_2, which is kept even lower by catalase and glutathione peroxidase). However, these reactions are also highly influenced by the iron chelator.

The final reaction to study is the oxidation of the biomolecule of interest. We generally think of the oxidation of lipid, protein or DNA. However, one could also consider the oxidation of the iron reductant, such as glutathione or ascorbic acid but including biogenic amines. The ligands of iron can be considered in a number of ways, such

as with strong chelators, physiologically or toxicologically relevant chelator (the nature of which are both unknown), or simply by the buffer used in in vitro studies. The most logical chelator in many studies where strong chelators are not used is simply the buffer. Many buffers (i.e., phosphate or HEPES) are excellent iron chelators. The reduction and oxidation of iron can be studied quite easily by using different reductants and oxidants in various buffers. Then the consequences of these reactions can be assessed by assay for the oxidation of a biomolecule.

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Synergy of iron in the toxicity and carcinogenicity of polychlorinated biphenyls (PCBs) and related chemicals

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Abstract

In Ah-responsive C57BL/10ScSn mice a single dose of iron significantly potentiated the property of the polychlorinated biphenyl (PCB) mixture Aroclor 1254 to induce porphyria by inhibition at the uroporphyrinogen decarboxylase stage of hepatic haem biosynthesis. The induction of liver tumors and other lesions were also enhanced markedly by iron overload suggesting a link between porphyria and cancer. The cellular, molecular and biochemical processes involved have been investigated in attempts to explain these phenomena by an iron-catalysed 'oxidative stress' mechanism.

Keywords: Iron; PCBs; Hepatic porphyria; Liver cancer

1. Introduction

Iron catalyses many fundamental reactions between oxygen and biomolecules due to its ability to switch between 2 stable oxidation states. For normal cell function the participation of iron is tightly controlled as a ligand of haemoproteins, iron sulphur proteins or proteins such as ribonucleotide reductase [1]. In vivo, most of the iron not in use is stored as ferritin. A small 'free' iron pool is thought to exist, although this is kept to a minimum due to the danger of unregulated redox activity. Evidence for such an iron pool has been difficult to obtain [2]. Many toxic, carcinogenic and pathological processes are now believed to be due, in part, to 'oxidative stress' occurring through the generation of reactive oxygen species (ROS) such as $O_2^{-\cdot}$. However, often the reactions of these species with lipids, proteins and DNA, probably only occur slowly without catalysis by the 'free' iron pool or that released from storage or transport proteins [3]. Redox cycling chemicals such as paraquat, diquat, and adriamycin release iron from ferritin in vitro, promoting lipid peroxidation and their effects can be protected against by iron chelators [2]. Much in vitro evidence implicates iron in the carcinogenic action of some asbestos-type fibres [4]. Considerably less is known about iron metabolism affecting potential toxic processes in vivo. In rodents, the development of chemical-induced colorectal and mammary cancer are adversely influenced by increased iron levels [5,6]. Ferric ethylenediamine-*N,N'*-diacetate and related chelates cause kidney tumors in rats and mice [7]. In humans, quite a

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lot of evidence links directly iron status with risks of neoplasia [8].

One of the most interesting findings of the influence of iron on the toxicity of chemicals is the involvement of iron in the induction of porphyria by polyhalogenated aromatic chemicals such as hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs) and tetrachlorodibenzodioxin (TCDD) [9]. The potentiating effect of iron on HCB-induced porphyria in rats was first shown by Taljaard et al. [10] and later its influence on TCDD-induced porphyria in C57BL/6J mice was demonstrated quite clearly by Sweeney et al. [11]. Iron overload was also shown to potentiate massively the induction of porphyria by HCB in C57BL/10ScSn mice [12]. Because there is substantial evidence linking iron to the development of porphyria cutanea tarda in humans [13], these findings with HCB and TCDD are well recognized in the 'haem' field but seem to have been of less interest to toxicologists. Probably of importance here is the general feeling that porphyria, though of interest to those in the field, has no wider connotations for mechanistic and risk toxicology. A crucial factor here would be the demonstration that iron status influences other aspects of the hepatic toxicity and also the hepatocarcinogenicity of HCB, PCBs and TCDD. In fact, iron overload has been shown to significantly enhance the hepatocarcinogenesis of HCB in female rats [14] and particularly in male C57BL/10ScSn mice [15]. We have also demonstrated that iron potentiates markedly the carcinogenicty of the PCB mixture (Aroclor 1254) in mice. Here we summarize these and more recent findings on the synergy between iron and PCBs and describe experiments exploring the mechanisms involved.

2. Materials and methods

Aroclor 1254 was a gift originally to Dr. J.B. Greig from Monsanto. HCB was purchased from BDH Chemicals Ltd (Poole, UK) and contained no detectable dioxins. All other chemicals were from Sigma-Aldrich Co. Ltd (Poole, UK).

Most mice were bred in the MRC Toxicology Unit but some were purchased from Harlan-Olac Ltd, Bicester, UK.

Mice were treated with iron by s.c. injection of iron-dextran solution (100 mg/ml Fe and 100 mg/ml dextran) at a dose of 600 mg Fe/kg or the equivalent dextran solution alone. After 3 days or 1 week mice were administered RM3 diets containing Aroclor 1254 (0.01%) and corn oil (2%). Mice were housed in negative pressure isolators for protection of staff. Maintenance of mice and experimental procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. At the end of experiments animals were killed by cervical dislocation or by perfusion under terminal anesthesia to obtain isolated hepatocyte preparations as described previously [16]. Ploidy studies of cells were conducted by flow cytometry in conjunction with assessment of nuclearity by histological techniques [16].

For biochemical studies livers were homogenized in O.25 M sucrose and nonhaem iron, porphyrins, and uroporphyrinogen decarboxylase levels were determined as reported before [12,14,15].

3. Results and discussion

3.1. Potentiation of both porphyria and liver cancer by iron overload

When male C57 Bl/10ScSn mice were injected with iron-dextran complex and then subsequently administered the PCB mixture Aroclor 1254 at 0.01% of the diet for 5 weeks, the induction of hepatic porphyria was greatly exacerbated compared with the equivalent group not given iron

Table 1
Inhibition of hepatic uroporphyrinogen decarboxylase (UD) and induction of uroporphyria in C57BL/10ScSn mice exposed to iron and Aroclor 1254 for 5 weeks

Diet	Iron	UD (pmol/min/mg)	Porphyrins (nmol/g)
Control	–	13.7 ± 0.6	0.6 ± 0.1
Control	+	14.3 ± 1.9	0.6 ± 0.1
Aroclor	–	15.9 ± 1.6	42 ± 21
Aroclor	+	5.8 ± 1.6*	630 ± 72*

*Significantly different from other groups at $P < 0.05$.

(Table 1). This porphyria was characterized by inhibition of the haem biosynthesis enzyme uroporphyrinogen decarboxylase in the liver and the accumulation of uroporphyrin I and III isomers (Fig. 1). Thus as we have previously reported with the weaker agent HCB [12], preloading mice with iron significantly sensitizes them to the development of uroporphyia caused by PCBs. The exact mechanisms causing the depression of uroporphyrinogen decarboxylase activity are not known but there is good evidence that an inhibitor of the enzyme is formed perhaps via oxidative attack of uroporphyrinogen [9].

In other studies with HCB in rats and mice, we proposed that iron should potentiate not only the porphyrogenic properties of this chemical but also its ability to cause liver tumors. This indeed was shown to be the case [14,15]. The demonstration that iron also enhances the porphyria induced by PCBs (at least Aroclor 1254) has led to the experiment to determine whether such pretreatment would also potentiate the hepatocarcinogenicity. This experiment was highly successful (Table 2). Thus iron will interact synergistically with HCB and PCBs in C57BL/10ScSn mice to cause both uroporphyria and liver cancer [17]. Of course it would be of great interest to determine whether iron overload will act in a similar way to alter the hepatic carcinogenicty of TCDD. In fact, there is preliminary evidence that this may be true (A.G. Smith, unpublished data). Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and β-naphthoflavone will also cause uroporphyria in iron-loaded mice [18], but interestingly, after 12 months of the latter in the diet at 0.05% in combination with iron overload, there was no development of liver

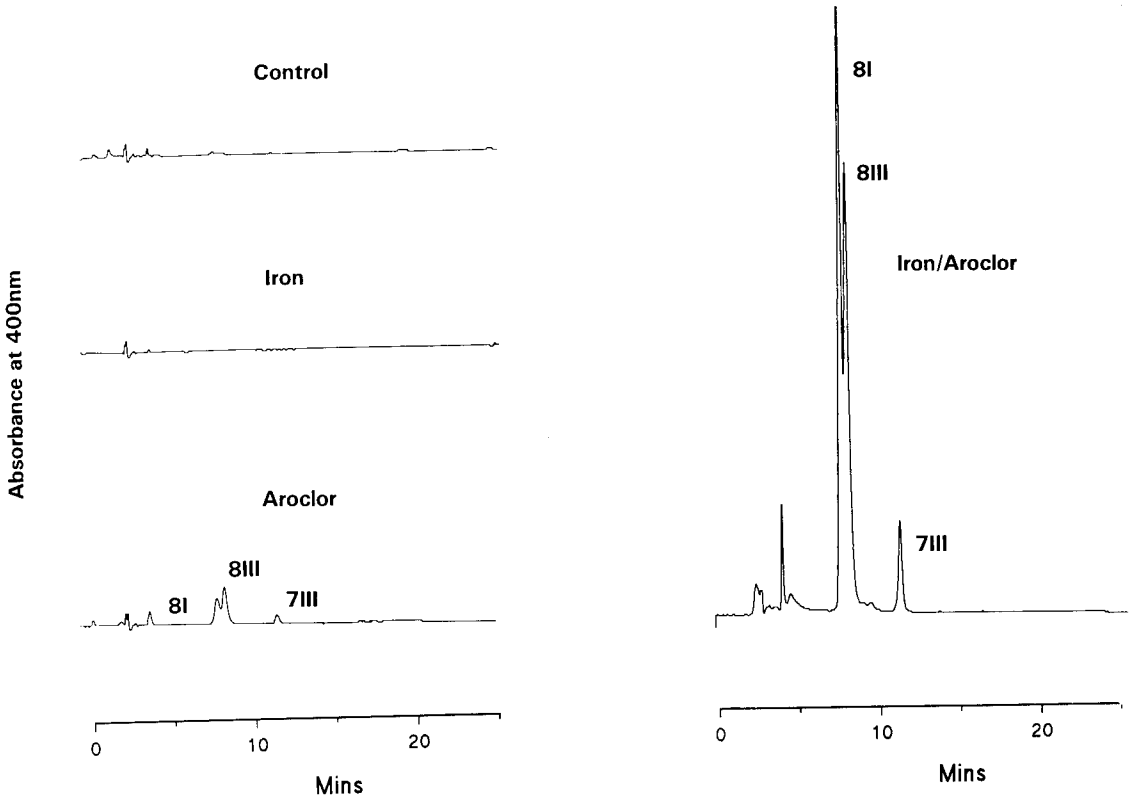


Fig. 1. Reverse-phase HPLC of porphyrins from livers of C57BL/10ScSn mice 5 weeks after administration of Aroclor 1254 diet with or without prior iron administration. 8I, uroporphyrin I; 8III, uroporphyrin III; 7III, heptacarboxylic porphyrin III. Samples represent equal amounts of liver and were detected on the same absorbance scale.

Table 2
Hepatic tumor incidence in C57BL/10ScSn mice administered iron and Aroclor 1254 for up to 12 months

Diet	Time (months)	Iron treatment	Survival (no./initial)	No. with nodules	No. with carcinomas
Control	8	–	10/10	0	0
Control	8	+	10/10	0	0
Aroclor	8	–	10/10	0	0
Aroclor	8	+	9/10	7	0
Control	12	–	15/15	0	0
Control	12	+	15/16	0	0
Aroclor	12	–	16/17	0	0
Aroclor	12	+	18/19	15	7

tumors (A.G. Smith, unpublished data). Phenobarbital (0.5%) in conjunction with iron also had no effect. It would seem that although there is a link between the potential of a chemical to cause liver tumors in mice and its iron-enhanced porphyrogenicity, there may not be a direct relationship.

3.2. Carcinogenesis in C57BL/10ScSn mice induced by iron-Aroclor1254 synergy

In the short term (2 months), besides uroporphyria, Aroclor 1254 and iron had a synergistic influence on liver weight in C57BL/10ScSn mice that was not observed with the DBA/2 strain which is resistant to both porphyria and tumor formation (Table 3). Centrilobular hepatocytes had enlarged nuclei and cytoplasmic vacuolation. There was also a synergistic increase in mitotic rate in the Ah-responsive C57BL/10SCSn strain not observed in the Ah-nonresponsive DBA/2 mice (Table 3). From 4 months onwards iron significantly potentiated oval cell and bile duct proliferation and cholangiofibrosis in C57BL/10ScSn mice administered Aroclor 1254. Basophilic nodules which emerged were more commonly associated with carcinoma formation than clear cell nodules.

Foci, nodules and carcinomas from iron/Aroclor mice were examined for the presence of mutations in the Ha-*ras* proto-oncogene at codon 61 using polymerase chain reaction (PCR) to amplify DNA from formalin fixed sections, followed by oligonucleotide hybridization. No mutations (0/28) were observed and only 2/23 for similar examinations of iron/HCB-induced tumors [19]. Examinations of transplantable tumor lines produced from either HCB- or Aroclor-induced tumors also showed no Ha-*ras* mutations at codon 61 or p53 mutations in exons 5, 7, and 8 by PCR-single-strand conformation polymorphism analysis (A.G. Smith and F. Gray, unpublished data). In many hepatocarcinogenic regimes in rodents, a characteristic mononucleated diploid population arises. Using flow cytometry, it was demonstrated that the synergistic interaction of iron with the PCB mixture

Table 3
Influence of iron and Aroclor 1254 diet (2 months) on liver weights and mitotic rates (no./1000 cells) in C57BL/10ScSn and DBA/2 mice

Strain	Diet	Iron	Liver % body weight	Mitotic rate
C57BL/10	Control	–	4.9 ± 0.1	<1
	Control	+	6.3 ± 0.7	<1
	Aroclor	–	6.4 ± 0.4	10 ± 3
	Aroclor	+	13.7 ± 0.6*	20 ± 3*
DBA/2	Control	–	5.2 ± 0.1	<1
	Control	+	7.1 ± 0.2	<1
	Aroclor	–	5.1 ± 0.2	<1
	Aroclor	+	7.6 ± 0.2	<1

*Significantly different from Aroclor group at $P < 0.05$.

caused the accelerated development of such a population by 2 weeks of Aroclor diet (control 4.8 ± 2.4, iron 6.4 ± 1.7, Aroclor 3.2 ± 1.2, iron/Aroclor 9.8 ± 1.9% of hepatocytes) and became highly significant by 6 months (iron/Aroclor 22.7 ± 5.4%). An interesting side observation was the marked degree of polyploidy of nuclei observed for iron overload alone so that 2 weeks after a single dose of iron to C57BL/10ScSn mice octoploid nuclei represented 22.7% of total hepatocyte nuclei compared to 7.8% in controls [16].

3.3. Biochemical basis for the synergistic effect of iron

It is quite clear that iron overload has a marked influence on the development of both hepatic uroporphyria and tumor development caused by HCB, PCBs and possibly TCDD in the Ah-responsive C57BL/10ScSn mouse but not in the nonresponsive DBA/2 strain. This could suggest that genes expressed via the Ah receptor are involved in the mechanism of toxicity. Current theories center on uncoupling of an induced cytochrome P450 1A system to create an iron-catalysed 'oxidative stress' [9] as shown in Fig. 2, which not only causes porphyria by oxidation of a haem precursor, but damages DNA. However, it must be said that evidence is not great so far. In fact, iron overload can depress cytochrome P450 levels. On the other hand, certain glutathione (GSH)-transferase activities and proteins are synergistically induced by the HCB-iron system in rats [14] and the iron-Aroclor system in mice (F. Mann and A.G. Smith, unpublished data). At the same time there is a loss of selenium-dependent GSH peroxidase activity which often occurs in oxidative environments. Probably the best evidence for an iron-catalysed oxidative mechanism is the synergistic enhanced occurrence of 8-hydroxydeoxyguanosine in the DNA from C57BL/10ScSn mice treated with iron/Aroclor but not in the DNA from similarly treated DBA/2 mice [20]. Further studies are in progress to elucidate the mechanisms.

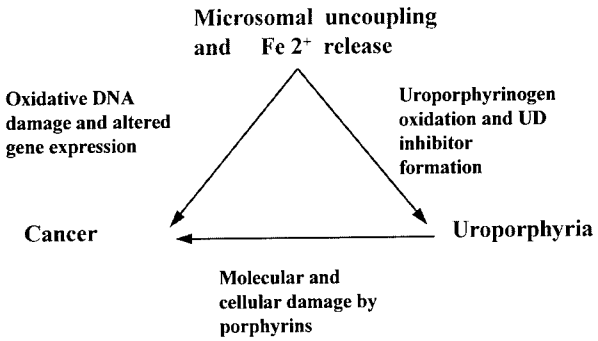


Fig. 2. Possible relationships between mechanisms of hepatic porphyria and carcinogenesis.

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Role of iron in the reactivity of mineral fibers

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Abstract

Any foreign body containing iron may be (or become) highly toxic in vivo. If its solubility in water is poor, surface chemistry governs the reactivity at the solid-liquid interface. Iron toxicity thus increases with the extent of exposed surface. Iron of endogenous origin may also be deposited on the particle surface and be activated under particular circumstances. The chemical processes that implicate surface iron as a primary cause of toxicity are: free radical release, mobilization by chelators, iron-catalyzed reactions. Three kinds of solids are compared: (i) well-known toxic materials, for example asbestos; (ii) non-toxic iron oxides; and (iii) model solids with surface exposed iron prepared for investigations on the reactivity of iron in biological media. The iron content of the solid is not directly related to the biological response: only a small fraction of ions, in a well-defined coordination and redox state, appears involved in the toxicity of the mineral dust.

Keywords: Asbestos fibers; Artificial fibers; Iron toxicity; Free radical release; Iron mobilization; Chelators; Phagocytosis

1. Introduction

The toxicity of iron in fibers is mostly confined to xenobiotic particulate trapped in the lower respiratory tract. It is well known that fibrous materials are more toxic than isometric particulate having the same chemical composition. The aspect ratio of the particle per se does not however justify the adverse biological response, which mostly depends on the chemical composition of the fiber itself. It is now generally accepted that both the form of the particle and its crystallochemical composition determine the ultimate toxicity [1–3].

The molecular mechanism whereby several mineral fibers – typically asbestos – originate serious diseases when inhaled is still unclear. Antioxidant enzymes and strong iron chelators decrease their toxicity in cell cultures and experimental animals, strongly suggesting that a crucial role might be played by iron-generated active oxygen species (AOS) [4–7].

The mere identification of one component in the mineral implied in the toxicity, however, is not sufficient to predict the extent of damage and the toxicity of particles of similar composition. A more complex mechanism than with water-soluble toxic substances takes place when toxicity is originated by solid/biological matter interactions. The toxic moiety, in the present case iron

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ions, has to be bioavailable in order to elicit a biological response. This depends on a large variety of factors related partly to the solid (redox state, crystallinity, micromorphology of the particle, location of the ions in the external layers) and partly to the biological compartments in which the fiber will be located (e.g. extracellular or intracellular medium, phospholipid lining layer, phagolysosome, etc). The interaction between the solid surface and the various biological compartments will modify both particles and tissues, and give rise to the pathogenic process.

The present paper deals with some physicochemical aspects related to the toxicity of iron in solids and to the possible prediction of the extent of damage caused by inhalation of iron-containing particulate.

2. Presence of iron in mineral and artificial fibers

Iron, being the most abundant metal on the earth's crust, is very often present, at least in traces, in most minerals [1,8]. We may classify fibers from the standpoint of their iron content in 3 main categories:

(1) Iron is a constitutive component of the mineral fiber as in crocidolite $[(Na^{+})_2(Fe^{2+})_3(Fe^{3+})_2(Si,A,Fe^{3+})_8O_{22}(OH)_2]$ or amosite $[(Fe^{2+}Mg)_7(Si_8O_{22})(OH)_2]$ both amphibole asbestos [1]. While both oxidation states – ferric and ferrous – occur in crocidolite only Fe(II) is present in amosite. This does not mean, however, that the amosite surface will only exhibit Fe(II) because spontaneous oxidation of surface ions is bound to occur in air.

(2) Iron substitutes for another ion, as in chrysotile asbestos ($[Mg_3Si_2O_5(OH)_4]$) where Fe^{2+} and Mn^{2+} replace Mg^{2+}. In such a case the quantity will vary from one specimen to another. Chrysotile asbestos is often associated with other iron-containing minerals, e.g. nemalite (a fibrous brucite, $Mg(OH)_2$ where iron replaces magnesium).

Erionite, a fibrous zeolite, regarded as the most potent carcinogenic mineral fiber [8,9], contains several metal ions, including occasionally iron, to compensate charges.

(3) Iron may also be present in artificial fibers as an undesired impurity. It often happens with rock and slag wools obtained from melt minerals; in traces it has also been found in glass wool [8]. In these cases the metal is not well dispersed over the fiber, but accumulates in some parts of it during manufacturing.

All iron in contact with atmospheric components tends to be oxidized to Fe(III). The rate of this process depends upon the location of the ion at the surface [10]. Heating accelerates oxidation so that artificial fibers obtained from the melt will be fully oxidized at the surface. Upon grinding, new Fe^{2+} ions are exposed and the newly formed surface becomes more reactive. Many cell-free tests have been in fact performed with freshly ground material [11]. In some cases grinding facilitates a rapid full oxidation, which decreases the material reactivity [12].

3. Fate of a fiber in vivo and stages in which the biovailability of iron plays a role

The inhaled fiber interacts with biomolecules, cells and tissues at different stages. The overall toxicity is the result of several subsequent events so that more than one single surface functionality may be implied [13]. Fig. 1 illustrates the possible

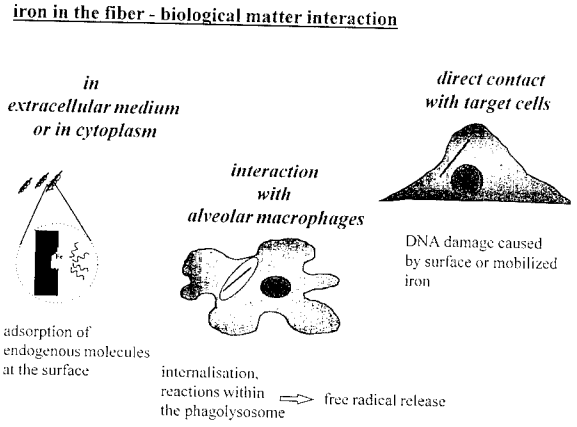


Fig. 1. Stage at which surface iron may react with fluids, cells and tissues.

role of iron in various circumstances. Adsorption of endogenous molecules at the metal ion site may determine the fate of the fiber. Interactions with cell membrane yield either membrane rupture and death of the cell or internalisation of the particle. Uptake by tracheal epithelial cells appears mediated by iron-derived AOS [14,15]. It is noteworthy that the presence of both oxidation states enhances the process [15]. The fiber may be phagocytized by activated alveolar macrophages in the attempt to attack and chemically destroy the foreign body, which, being an inorganic solid, is not liable to be demolished as any bacteria (Fig. 2). Phagocytosis will then be successful if the fiber is engulfed and cleared out of the lung. Long thin fibers are often partly internalized, i.e. with a part protruding out of the cell (frustrated phagocytosis), with consequent leaking of intracellular fluids; some smaller ones are internalized but cause internal membrane rupture and death of the cell (failed phagocytosis). In both cases, a direct contact between intracellular fluids (e.g., lysosomal content at low pH, in a strongly oxidizing medium) and the mineral surface will take place, with consequent redox and radical reactions, modification of enzymes, etc. Subsequent cycles of phagocytosing unsuccessful events may, on the one hand, modify the mineral surface itself, and, on the other, provoke a prolonged release of free radicals, mainly AOS, out of the cell, in the surrounding medium. All

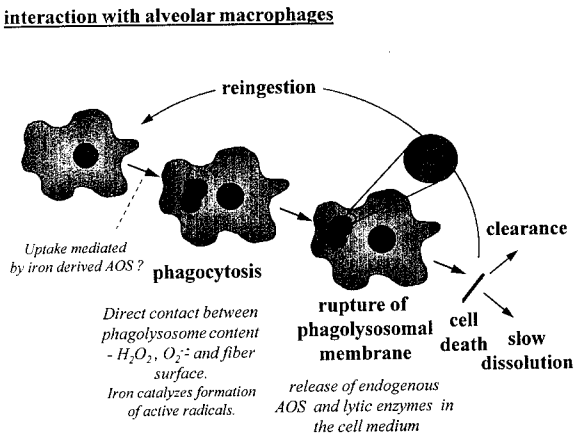


Fig. 2. Interaction between inhaled fibers and alveolar macrophages.

mineral dusts elicit an enhanced generation of free radicals from phagocytes: the intensity of this response, however, depends largely upon their crystallochemical features [16]. A close relationship between AOS production and cytotoxicity of the fibers (cytotoxicity index) was reported for a series of mineral and artificial fibers [17].

A consistent fraction of the inhaled fibers and particles are not cleared out of the lung, some are retained and coated by bioinorganic matter, originating the so-called ferruginous bodies. They are made up of a generally segmented coating of iron oxide of endogenous origin, whose significance and function is still uncertain [18]. It has been recently shown [19] that iron mobilized by low-molecular-weight chelators from these bodies may damage DNA, i.e., iron is still redox active. The formation of these bodies, mainly originated by deposition of ferritin, is strictly dependent on surface affinity for this protein. The segmented appearance is likely caused by deposition at specific surface sites [18]. Thus surface chemistry also regulates this peculiar phenomenon of biomineralisation.

4. How fibers and cells give rise to free radicals

There is much evidence from in vivo and in vitro tests supporting the hypothesis that free radicals and other reactive oxygen species are an important mechanism by which asbestos and other mineral fibers mediate tissue damage [4–7,20]. Evidence was found that antioxidants markedly reduced the pathogenic effect of asbestos. The mechanism whereby the fiber promotes or catalyzes the abnormal release of AOS is still under debate but several data indicate that a crucial role is played by the iron ions at the surface of the fiber. Addition of desferrioxamine, a potent iron chelator, decreases both cell damage and lipid peroxidation [20,21].

If iron and AOS are implied in asbestos toxicity, the question arises of what is the role of iron at the molecular level and whether AOS are of exogenous or endogenous origin or both.

In dust-cell interaction there are 2 possible

sources of free radicals: during phagocytosis various chemicals are produced by the cell in the attempt to destroy the foreign body, among which the superoxide anion, $O_2^{\cdot -}$ and hydrogen peroxide; the particle per se triggers radical reactions when in contact with body fluids.

In both cases the radical concentration attained may impair the body defenses and consequently radicals may reach some target cell and initiate a pathogenic process. The capability of a given material to release free radicals is thus of paramount importance in predicting its toxicity.

Three possible ways for free radical release from aqueous suspensions of mineral dusts (cell-free tests) have been envisaged so far [22]:

(1) Production of $^{\cdot}OH$ in presence of hydrogen peroxide, following a Fenton-type reaction

$$M^{n+} + H_2O_2 \rightarrow M^{n+1} + {}^{\cdot}OH + OH^-$$

first proposed for asbestos [23] and subsequently also for glass fibers [24] and for various other minerals such as kaolin in the presence of reducing agents [25,26]. It applies only to possible reactions occurring in well-defined biological compartments where hydrogen peroxide is present, typically the phagolysosome.

(2) Production of $^{\cdot}OH$ from O_2 present in the solution, following the reaction sequence:

$$O_2 + e^- \rightarrow O_2^-$$

$$O_2^- + e^- + H^+ \rightarrow H_2O_2$$

$$H_2O_2 + e^- \rightarrow {}^{\cdot}OH + OH^-$$

Mechanism 2 was proposed for a large variety of mineral and artificial fibers [11,27,28]. It may occur in any biological compartment as oxygen is ubiquitous, but requires efficient electron donating sites at the surface of the particle.

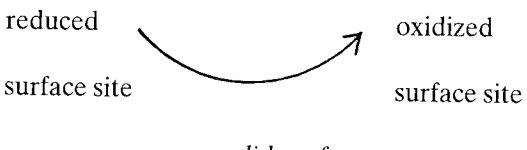
(3) Hydrogen abstraction from an organic molecule HR

$$X + H\text{-}:\text{-}R \rightarrow XH + R^{\cdot}$$

This mechanism does not necessarily involve AOS, although it does not take place in oxygen-free solutions. It appears the most general one and has been found on a larger variety of fibers [22,29–31] and iron minerals [32].

We have recently found in our laboratory that mechanisms 1 and 3 may occur on the same particle, but involve different kinds of active surface sites [22]. The 2 mechanisms operate with different target molecules and cause oxidation (a) and reduction (b) of the active surface site.

Mechanism 1:



$$H\text{-}O\text{-}O\text{-}H \longrightarrow OH^- + {}^{\cdot}OH$$

Mechanism 3:



$$HCOO^{\cdot} \longrightarrow CO_2^{\cdot -}$$

The 3 mechanisms apply to aqueous suspensions of the solid particles and involve interfacial phenomena. Iron implicated may be strictly bound to the surface, mobilized at the interface layer or even into the solution by chelators (see below). In the experimental conditions reported for the evaluation of free radical release (spin trapping) the only possible chelator is the phosphate ion from the buffer. Investigations on the activity of the filtrate by us and other authors [12,31] revealed that the effect of iron in solution is minimal if any.

Not all iron-containing minerals are toxic [33], similarly not all iron-containing minerals release free radicals in solution [34,35]. The case of different categories of iron-containing mineral particles is illustrated in Table 1:

- simple iron oxides (hematite (Fe_2O_3) and magnetite (Fe_3O_4);
- asbestos: (i) the 2 most widespread (chrysotile (UICC B), and crocidolite); (ii) 2 asbestiform minerals from Piedmont ores (Italy)

Table 1
Iron content, surface area and free radical release

Solids	Specific surface (m^2/g)	Iron content (% W/W)	$R^{\cdot}$ radical release[a] $H^{\cdot}$ abstraction (a.u.)		$^{\cdot}OH$ radical release[a] (a.u.)	
			Aged	Freshly ground	Aged	Freshly ground
Hematite	3	70	Blank	Blank	Blank	Blank
Magnetite	27	72	50	n.d.	135	n.d.
Chrysotile	27	2–6	275	580	223	190
Crocidolite	8	27	83	300	112	160
Balangeroite	3	4–6	40	100	100	130
Carlosturanite	4	5–7	30	480	250	205
FeY zeolite	n.d.	4	2500	n.d.	125	n.d.
FeO/MgO	40–50	5	1380	1460	160	185

[a] Free radical release was detected by means of the spin trapping technique, employing DMPO as spin trap. In both cases the intensity of the EPR signal measures the number of radicals trapped as [DMPO-$CO_2^{\cdot -}$] adduct (formate ion as target molecule) for $H^{\cdot}$ abstraction and as (DMPO-$OH^{\cdot}$) adduct (hydrogen peroxide as target molecule) for $^{\cdot}OH$ radical release [22].

(balangeroite and carlosturanite, the former being a typical contaminant of chrysotile from Balangero (Italy). In both iron is a constitutive component [36];

- 2 model solids prepared for investigations on the toxicity of iron-containing materials [22]. An iron exchanged Y zeolite (FeY, ionic exchange performed in a solution of $FeSO_4$) [22] and a solid solution of ferrous and magnesium oxide (FeO/MgO). The former mimicking the silicic framework, the latter the brucitic part of asbestos.

Iron content and specific surface of each solid are compared with their capability to release free radicals following mechanism 1 ($^{\cdot}OH$ from H_2O_2) or 3 ($H^{\cdot}$ abstraction) evaluated by means of the spin trapping technique [22]. The extent of free radical release does not parellel iron content, nor surface iron. The oxides are the richer in iron and the most inactive, whereas model solids, which are relatively poor in iron but with ions poorly coordinated and mostly in low valency state, are very reactive, particularly in H abstraction.

This is in agreement with what was previously found by Pézerat and coworkers over a wide variety of iron-containing minerals, some active and some inactive [29–32]. The propensity to yield H abstraction was associated to the presence of Fe(II) 'accessible' at the mineral surface [37]. Accessibility was evaluated through chelation of Fe(II) by 2,2′-bipyridyl in phosphate buffer and formation of the stable iron-bipyridyl complex in solution. As this mechanism – at least in vitro – is a non-catalytic one [22], the amount of radicals released necessarily reflects the extent of active sites at the surface. The chemical nature of it is still under debate: an association between Fe(II)–Fe(III) (couples or small clusters of Fe^{2+}-Fe^{3+}) [10,13,22,35] or ferryl/perferryl groups, originated by oxidation in air of Fe(II) [29–32] appear the best candidates. The former somehow recalls what was found for the initiation of lipid peroxidation in homogeneous media [38].

Mechanism 1 appears somehow insensitive to the content of iron; it takes place on all solids, except hematite, with relatively similar intensity, the most active (carlosturanite) releasing just twice the amount of radicals than the least active ones (crocidolite and Fe-zeolite). Few active sites catalyze the reaction: glass fibers with just traces of iron have been found more active than crocidolite itself [24]. The coordination state of iron in glass fibers is such that the iron becomes catalytic.

Table 1 also illustrates the effect of newly formed surfaces – freshly ground vs. aged samples – on free radical potential. It is well known that mechanical grinding activates the surfaces and its impact on the toxicity of inhaled dusts has been stressed by several authors [10,13,39,40]. Grinding is effective on mechanism 3, particu-

larly with asbestos, but not on mechanism 1. This points to an involvement of 'not yet oxidized' Fe(II) at the new surface in mechanism 3 which is ineffective on mechanism 1.

If mechanism 3 is the one related to the pathogenic activity of iron in minerals as postulated by Pézerat and coworkers [29–32,41], a question arises on the fate of the surface active sites in vivo. May active sites enter a redox cycle whereby a continuous release of free radicals takes place? Fig. 3 reports the [DMPO-$CO_2^{\cdot}$] electron paramagnetic resonance (EPR) spectra obtained with the FeY zeolite pretreated with an excess of hydrogen peroxide and subsequently with glutathione. The surface is inactivated by hydrogen peroxide (fully oxidized to ferric iron) but glutathione largely restores its activity. Similar activation/inactivation cycles may occur during successive failed phagocytosis (Fig. 2).

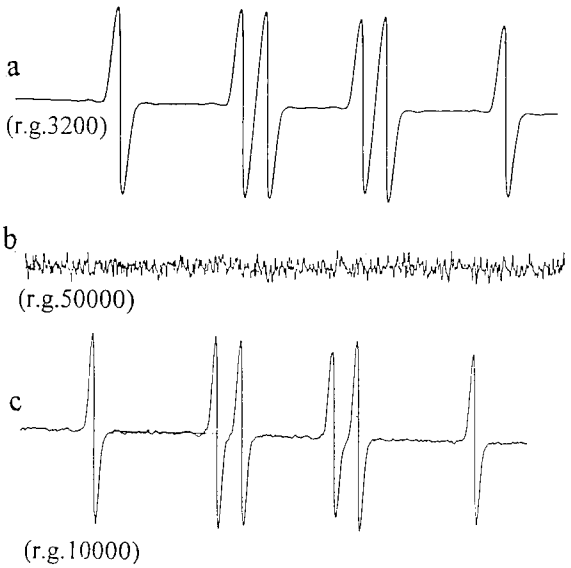


Fig. 3. EPR spectra of the DMPO-$CO_2^{\cdot -}$ adduct produced in a suspension of FeY in a buffered solution (pH 7.4) containing sodium formate (0.5 M) and DMPO (0.05 M) incubated 30 min at 37°C. EPR experimental conditions were: microwave power 10 mW; modulation frequency 100 MHz; modulation amplitude 1 G; scan range 100 G; field set 3380. (a) Original FeY; (b) FeY incubated in H_2O_2; and (c) FeY ex H_2O_2 incubated in glutathione.

5. Biochemical reactions relatable to the presence of iron in the fiber

5.1. Lipid peroxidation

Asbestos can catalyze lipid peroxidation in cell membrane lipids or in model systems such as fatty acid emulsions or liposomes [7,21,42–44]. Iron-catalyzed reactions via radical pathways are suggested, as AOS scavengers and desferrioxamine inhibit the reaction. The process of free radical formation detected with the spin trapping technique, however, does not appear to be correlated to lipid peroxidation. The 2 processes have common characteristics as they both depend on iron ions available in the mineral and require ferrous ions [37,45] but a comparative analysis of the results obtained on the same group of iron-containing minerals revealed different activities of the minerals in the 2 tests [44]. This is in agreement with findings in homogeneous systems. Lipid peroxidation induced by soluble iron complexes is dissociated from the pathway leading to free hydroxyl radical generation in liver microsomes [46] and the same was observed for lipid peroxidation and oxy-radical formation induced by ferritin [47]. It is also noteworthy that the toxicity to macrophages was found to be not related to crocidolite asbestos-induced lipid peroxidation [44].

It has been recently reported that fiber-induced lipid peroxidation depends both on the composition of the incubating solution (mainly presence of chelators) and on the texture of the minerals involved. These factors may also explain why some fibers induce lipid peroxidation in linolenic acid but not on epithelial cell membranes [31].

5.2. DNA damage

DNA damage caused by asbestos has been investigated in various ways. Production of 8-hydroxydeoxyguanosine was detected both in the presence [48] and in the absence [28] of hydrogen peroxide. In spite of the fact that the $OH^{\cdot}$, as a short-life radical, is expected to be unable to

reach the target cell, direct hydroxylation of DNA by OH˙ originated by asbestos was found by Leanderson et al. [28]. A mineral-mediated oxidation yielding purine decomposition products has been obtained directly from incubation of minerals with nucleosides [49].

The induction of single strand breaks (SSB) in DNA by various asbestos was largely investigated by Aust and coworkers [50–53]. They have concentrated their work on the iron that may be mobilized from asbestos by low-molecular-weight chelators, pointing out that in that form iron may reach the target cell, particularly DNA, more easily than iron retained on asbestos. Considerable amounts of iron were extracted from crocidolite and other asbestos by endogenous chelators and it was clearly demonstrated that the extent of DNA damage parallels mobilized iron. Hydrogen peroxide and ascorbate increase the induction of SSB, suggesting a redox radical mechanism involving iron in low oxidation state. Both solid surface and mobilized iron may trigger the relevant radical reaction. Considering, however, that the fiber has to be close to the target biomolecule in order to catalyze damage reactions, target cells will likely be more easily reached by mobilized iron. A synergistic effect of surface and chelated iron in solution may also be considered when dealing with iron toxicity in vivo.

6. Mobilization of iron at the solid/liquid interface

6.1. Iron depletion

Removal of iron from various asbestos by incubation in several iron chelators, endogenous and exogenous, was widely investigated by Lund and Aust [50–52]. In spite of the minimal solubility of the mineral fiber in water, the presence of chelators induced a substantial release of iron in solution. Association of a reductant and of a chelator appear the most effective agents [52,54].

Iron released does not parallel the mere iron content of the mineral [34] but depends on several factors including crystalline structure, surface area, coordination of the ion and redox state. By comparing the results obtained on a set of fibers by a set of chelators, it has been recently found in our laboratory that the potential for iron abstraction of a chelator is also a function of the solid involved, e.g. the most effective chelator for crocidolite is not necessarily the most effective one for amosite. Surface chemistry thus also regulates phenomena relative to soluble iron. Successive incubations revealed that within the solid, iron depletion induced substantial ion mobility [35] so that it becomes difficult to define a true equilibrium state. Mobilization rates measured in the same conditions enable correct comparisons of the behaviour of different solids. Fig. 4 reports the amount of iron removed by desferrioxamine from 2 chrysotiles (UICC A and B), crocidolite (UICC) and nemalite, a fibrous brucite associated to Canadian chrysotile [10,11,31].

Iron release is immediate from nemalite whereas depletion from the 2 asbestos in the first 2 h is relatively weak but then depletion kinetics remarkably increase. While at the beginning iron removed from nemalite is more than 10-fold that which was removed from the 3 asbestos at the

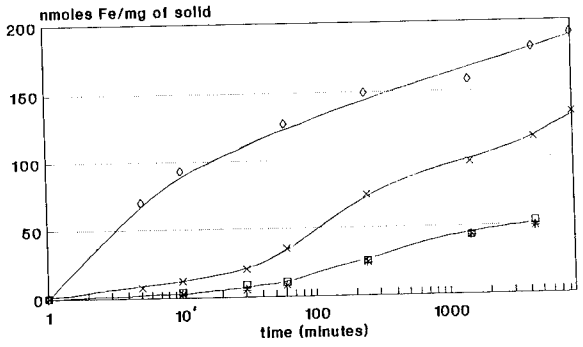


Fig. 4. Kinetics of iron mobilization from some asbestos by desferrioxamine. Suspensions of asbestos (10 mg asbestos/1 ml chelating solution) were incubated at 37°C. The iron amount in the supernatant solutions was evaluated at different times by visible spectroscopy (428 nm, Fe^{3+}-desferrioxamine complex). Nemalite, ◇; crocidolite, ×; chrysotile A, *; chrysotile B, □.

end of the experiment (7 days of incubation), the amounts released are about 50, 130 and 180 nmol per mg of the solid for chrysotile, crocidolite and nemalite, respectively.

Incubation in various chelators did not modify the crystalline structure of asbestos as far as their detectability by X-ray diffraction is concerned but micromorphology and organisation of the outmost layers, iron coordination and potential for free radical release were seriously affected [22,35]. Electron microscopy (high resolution electron microscopy) revealed that following iron depletion the crystallinity of the external layers was lost in amosite and crocidolite fibers [35]. Fibers pretreated with the same chelators used by Lund and Aust for selective removal of Fe^{3+} and Fe^{2+} (desferrioxamine and ferrozine) fully inhibited radical release following hydrogen abstraction (mechanism 3) confirming that both oxidation states are required for this reaction [22].

6.2. Iron deposition

Fibers depleted from iron may reuptake iron from solutions. A coating of Fe(III) was reported to inactivate free radical release from crocidolite asbestos [55]. The mechanism is likely the same whereby hydrogen peroxide inactivates the fibers, i.e. ferric iron alone is non-reactive. Any kind of ion, including ferrous or ferric, may be deposited at the surface thus potentially modify its toxic potency. Iron deposition was found to activate erionite in the induction of DNA SSB [56] to increase the ability of crocidolite in the same reaction [57].

A peculiar case of deposition is the formation of ferrouginous bodies. Whether they are the product of a defense of the organism from the fiber or not, they certainly contain considerable amounts of iron that may become potentially toxic under some circumstances. It has been recently shown that iron associated with these bodies is responsible for the formation of SSB in DNA, and that this effect was increased in the presence of low-molecular-weight chelators [18].

A complex cycle of iron depletion-iron deposition will take place in vivo, whereby endogenous chelators will remove iron while ferritin and/or hemosiderin will be deposited as a coating on the fiber. Far from being unchanged the inorganic fiber in a biological environment will thus undergo continuous surface modifications, determining the ultimate toxicity of the inhaled fibers.

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Toxicology Letters

Potential protection from toxicity by oral iron chelators

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Abstract

The design of clinically useful iron chelators requires attention to be paid to 3 key parameters: oral absorption, selectivity and affinity for iron(III) and toxicity. Factors which influence these 3 parameters are discussed. Hydroxypyridinones are identified as key ligands and properties leading to minimal toxicity and optimum distribution for the treatment of thalassaemia are presented. Key metalloenzymes which are inhibited by iron chelators are identified.

Keywords: Iron chelators; Oral activity; Metalloenzymes; Hydroxypyridinones; Cell cycle

1. Introduction

The design of an orally active, non-toxic, selective iron chelator has been a goal of many medicinal chemists over the past 20 years. Naturally occuring siderophores provide excellent models for such molecules, indeed, desferrioxamine has been and still remains an extremely useful iron chelator. Unfortunately, it lacks high oral bioavailability. Many other siderophores have been investigated but all have failed under in vivo conditions for one reason or another [1]. Consequently a radical approach was required to tackle this most difficult problem. We have simultaneously monitored 3 different parameters in an attempt to design useful iron chelators. These are oral absorption, selectivity and affinity for iron, and toxicity. Although an ideal orally active, non-toxic, iron chelator has yet to be identified, we believe that we are well on the way to solving the problem. In this overview the critical parameters outlined above will be discussed and recent developments with hydroxypyridinone chelators described.

2. Absorption of chelators from the gastrointestinal tract

Two major factors influence the non-facilitated absorption of a drug from the gastrointestinal tract, the oil/water distribution coefficient and the molecular weight. Whereas there is considerable quantitative information concerning the distribution coefficient [2], there are remarkably few studies devoted to the effect of molecular weight. Non-facilitated diffusion is generally considered to be dominant for drugs with molecular weights <200. The transcellular route involves diffusion into the enterocyte and thus utilises some 95% of the surface area of the small intestine. In contrast, the paracellular route only utilises a small fraction of the total surface area and the corresponding flux via this route is much

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smaller. The cut-off molecular weight value for the paracellular route in the human small intestine is approximately 400 [3] and that for the corresponding transcellular route, as judged by polyethylene glycol permeability is 500 [4]. However with iron chelators it is essential to achieve efficient absorption from the gastrointestinal tract, as daily doses probably need to fall within the range 1–2 g in order to maintain negative iron balance. Thus 50% absorption of the dose would leave 1–2 g remaining in the lumen of gut – a level which might be expected to cause disturbance to the microbiological flora. In order to achieve greater than 70% absorption the chelator molecular weight probably needs to be less than 300 [4].

This molecular weight limit places a considerable restriction on the choice of chelator and effectively excludes hexadentate ligands from consideration. Most siderophores, including desferrioxamine have molecular weights between 550 and 900. Although EDTA has a molecular weight of 292, it is too small to completely encompass the chelated iron. DTPA (molecular weight 393) and HBED (I) (molecular weight 386) are probably close to the minimal size possible for effective hexadentate iron(III) ligands. Desferrioxamine (4) possesses a molecular weight of 561.

The absorption of both these ligands is known to be low, although the prodrug (dimethylester) form of HBED (I) has been reported to possess acceptable oral bioavailability [5]. In sharp contrast, by virtue of their much lower molecular weights, bidentate and tridentate ligands (Fig. 1) are predicted to possess higher absorption efficiencies. Thus the hydroxypyridinones, defer-

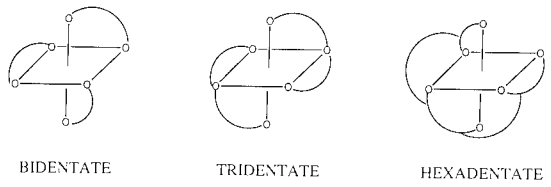
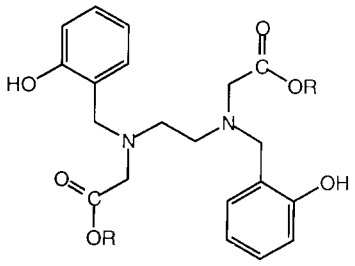
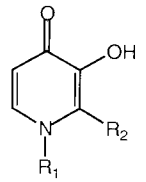


Fig. 1. Schematic representation of octahedral iron(III) complexes with bidentate, tridentate and hexadentate ligands.



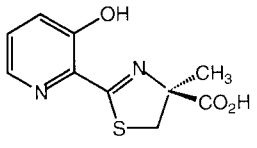
1

HBED (R = H)
HBED Dimethyl ester (R = CH_3)

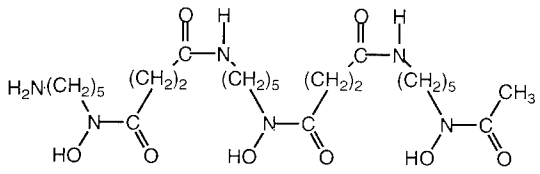


2

	R_1	R_2
CP20	CH_3	CH_3
CP94	C_2H_5	C_2H_5
CP102	$(CH_2)_2OH$	C_2H_5



3



4

Scheme 1. Structure of iron(III) chelators.

riprone (CP20) and CP94 (2), with respective molecular weights of 139 and 167, are both efficiently absorbed by man [6,7]. In similar fashion the tridentate chelator desferrithiocin (3), (molecular weight 238) is also efficiently absorbed from the mammalian intestinal tract.

3. Selectivity and affinity for iron

Iron chelators can be designed to be selective for either the iron(III) or iron(II) oxidation states. However all iron(II)-favouring ligands also possess appreciable affinities for the physiologically important zinc(II), copper(II), manganese(II) and cobalt(II) ions and thus the design of a non-toxic iron(II)-selective ligand is not possible. In contrast, although iron(III)-favouring ligands also possess favourable affinities for other tripositive cations, such as aluminium(III), gallium(III) and indium(III), these are not physiologically important. Thus in principle, it is possible to design a non-toxic iron(III)-selective ligand. An additional advantage of high affinity iron(III) ligands is that under aerobic conditions they will also chelate iron(II) cations and autoxidase them to the iron(III) species [8]. Thus, high affinity iron(III)-selective chelators bind both iron(III) and iron(II) under most physiological conditions.

Iron(III) forms most stable bonds with oxygen-containing chelating agents. It is for this reason that the majority of siderophores utilise the dioxo ligands catechol and hydroxamate. The affinity of such compounds for iron(III) reflects the pK_a values of the chelating oxygen atoms, the higher the affinity, the higher the pK_a value (Table 1) [9]. Thus catechol possesses the highest pK_a value followed by 3-hydroxypyridin-4-one; both chelating oxygens on these 2 ligands possess appreciable affinities for protons, in contrast to the other chelators listed in Table 1. The surprisingly high pK_a value of the carbonyl function of the 3-hydroxypyridin-4-one results from extensive delocalisation of the lone pair associated with the ring nitrogen atom. Although the catechol derivative possesses a higher β_3 value than that of the 3-hydroxypyridin-4-one, the corresponding pM value is lower. This difference is due to the relatively higher affinity of catechol for protons. Thus on the basis of pM values, pM is $-\log[Fe^{3+}]$ at pH 7.4 when $[Ligand]_{Total} = 10^{-5}$ M and $[Fe^{3+}]_{Total} = 10^{-6}$ M, the 3-hydroxypyridin-4-one class possesses the greatest affinity for iron(III). All the ligands presented in Table 1 possess the following relative affinities Fe(III) $\gg$ Cu(II) > Zn(II) $\gg$ Ca(II) > Mg(II).

Table 1
pK_a values and affinity constants for dioxobidentate ligands for iron(III)

	pK_{a1}	pK_{a2}	β_3	pM
Catechol	8.4	12.1	40	15
3-Hydroxypyridin-4-one	3.6	9.9	37	20
3-Hydroxypyridin-2-one	0.2	8.6	32	16
3-Hydroxypyran-4-one	–	8.7	28.5	15
1-Hydroxypyridin-2-one	–	5.8	27	16
Acetohydroxamic acid	–	9.4	28.3	13

pM = $-\log [Fe^{3+}]$ when pH = 7.4, $[Ligand]_{Total} = 10^{-5}$ M, and $[Fe^{3+}]_{Total} = 10^{-6}$ M.

4. Toxicity

Although bidentate and tridentate ligands possess a clear advantage over hexadentate ligands

Table 2
Comparative toxicities of iron(III) chelators

Bidentate and tridentate ligands	Hexadentate ligands
Penetration of BBB is dependent on K_{part} value	Generally low penetration of BBB
Kinetically labile – iron redistribution is possible	Kinetically inert – iron redistribution is unlikely
Affinity for iron is concentration dependent	Affinity for iron is concentration independent
Form 2:1 and 1:1 complexes which could be toxic	Only form 1:1 complexes, which are generally non-toxic

with respect to oral bioavailability they are potentially more toxic as is indicated in Table 2. Concerning the blood brain barrier (BBB), non-facilitated penetration of drugs is critically dependent on the distribution coefficient and values below 0.05 result in extremely poor penetration [10]. Thus if bidentate pyridin-4-ones are used with a distribution coefficient lower than this critical value, then entry to the central nervous system is predicted to be low. Problems associated with both the kinetic lability and concentration dependence of 3-hydroxypyridin-4-one (2) and desferrithiocin (3) iron complexes are minimal under physiological conditions due to the relatively high affinity of the ligands for iron(III) [9].

Hydroxypyridin-4-ones and desferrithiocin are efficient scavengers of iron even in the ligand concentration range of 1–10 μM [9]. As long as the 3:1 pyridinone and the 2:1 ferrithiocin iron complexes dominate the speciation of the low-molecular-weight forms of iron, then minimal hydroxyl radical production occurs. Indeed, the viability of cultured hepatocytes, as judged by the release of lactate dehydrogenase is enhanced by the presence of 3-hydroxypyridin-4-ones in the concentration range 2–100 μM [11].

One of the problems with orally active compounds such as the hydroxypyridinones and desferrithiocin is that by virtue of their relatively low molecular weight and favourable distribution coefficients, they rapidly penetrate most cells and hence gain access to a wide range of metalloenzymes.

4.1. Inhibition of iron-dependent enzymes

In principle chelating agents can inhibit metalloenzymes via 3 different mechanisms, removal of the essential metallic cofactor, formation of a stable ternary complex or deprivation of the apoenzyme of its normal source of metal ion. Iron(III) chelating agents generally do not inhibit haem-containing enzymes or iron-sulphur cluster proteins. They do however interfere with mono-iron and bi-iron centres coordinated to oxygen ligands. Examples of such enzymes are given in Table 3.

Table 3
Cu- and Fe-containing enzymes which are inhibited by iron chelators

Lipoxygenase family (5-, 12- and 15-)
Post translational modification of proteins
Prolyl-4-hydroxylase
Lysine-4-hydroxylase
Deoxyhypusyl hydroxylase
Aromatic hydroxylase family
Phenylalanine, tyrosine and tryptophan hydroxylase
Ribonucleotide reductase

In general lipoxygenases are inhibited by hydrophobic chelators [12] and therefore the introduction of hydrophilic characteristics tends to minimise inhibitory potential. Hydroxylation of specific protein residues is achieved by a closely related group of proteins each of which being selective for the -GlyXYGly motif. High chelator concentrations are generally required to cause appreciable inhibition; the IC_{50} values for CP20 (2) for instance are prolyl-4-hydroxylase (150 μM) and deoxyhypusyl hydroxylase (90 μM) [13]. Thus if the clinical levels of chelator can be limited to <25 μM, inhibition of these enzymes should not present serious problems. The aromatic amino acid hydroxylases are particularly susceptible to inhibition by hydroxypyridinones due to the similar molecular characteristics of the inhibition and substrate. However the introduction of an alkyl function at the 2 position of hydroxypyridinones markedly increases the K_i value to the mM range, presumably due to steric interference of the chelation process at the enzyme active site [14].

Inhibition of ribonucleotide reductase, in contrast to the previously discussed enzymes, presents a critical problem – depletion of deoxynucleotide pools, thereby inhibiting DNA synthesis by blocking elongation. Inhibition of ribonucleotide reductase therefore interferes with the G1 $\rightarrow$ S transition, thereby blocking the cell cycle. It is likely that the bone marrow toxicity induced by certain iron chelators results from the

inhibition of this enzyme [15]. Agranulocytosis and neutropenia have both been associated with high doses (>80 mg kg^{-1}) of CP20 [16].

Ferrochelatase can also be considered as an iron-dependent enzyme and may be subject to similar control mechanisms to that of the iron response protein (IRP) in that it possesses an iron-sulphur cluster [17]. Hydrophobic hydroxypyridinones inhibit this enzyme leading to protoporpharia, however hydrophilic chelators, for instance CP20 and CP102, lack such inhibitory activity (A. Smith, pers. commun.).

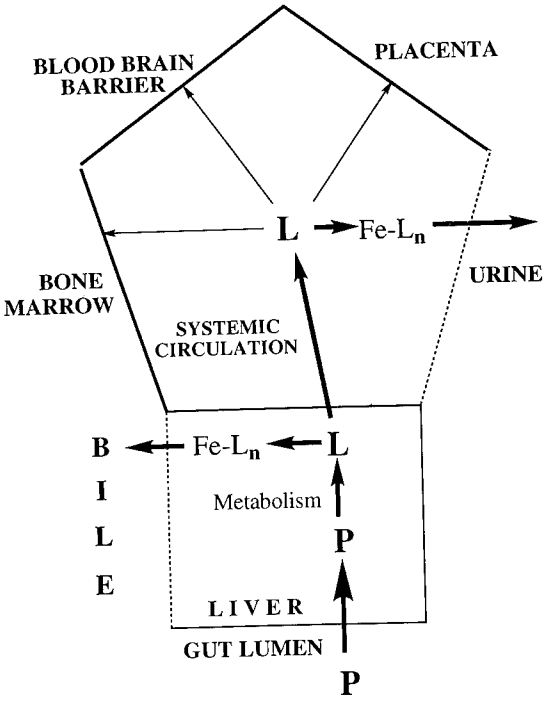


Fig. 2. Major fluxes of a chelator prodrug (P) after oral administration. If P is lipophilic (dist. coeff. >2), it will be rapidly cleared from the portal blood stream by the liver (1st pass kinetics). The prodrug can be designed such that it is virtually quantitatively converted, in the liver, to a more hydrophilic metabolite (L), which itself is not subjected to further metabolism. L chelates iron, the resulting complexes being secreted into the bile. Any non-chelated metabolite (L) will efflux into the systemic circulation, where it gains access to the non-transferrin bound iron pool. Again, any resulting iron complex will be excreted in the urine. If the dist. coeff. of L is adjusted to be less than 0.001, the molecule will not penetrate either the BBB or the placenta.

4.2. Distribution of chelators

As there are a number of enzymes and cell responses (for instance those dependent on IRP [18]) which are dependent on intracellular chelator levels, a practical strategy to minimise toxicity is to limit the distribution of the chelator to the region of elevated iron levels. Thus with thalassaemia major the liver is one of the major sites of iron overload, with Parkinson's disease it is the substantia nigra. An approach which is currently being developed for the treatment of thallassaemia involves the prodrug concept (Fig. 2) where the prodrug P, by virtue of its molecular weight and distribution coefficient is rapidly absorbed from the gastrointestinal tract and is also efficiently extracted by the liver. In the hepatocyte, the prodrug is rapidly converted to the iron-selective chelator L, which possesses a much lower distribution coefficient. The chelator L is capable of scavenging iron in the hepatocyte, but also can efflux into the systemic circulation, thereby scavenging the extracellular iron pool [19]. By virtue of the low distribution coefficient, the chelator, L, does not gain ready access to other cells, nor does it cross the BBB.

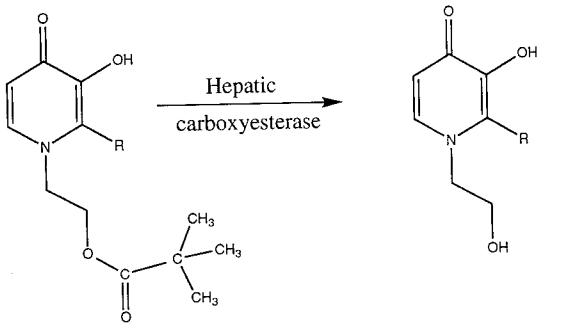


Fig. 3. Phase 1 metabolism of a pivoyl ester containing hydroxypyridinone to the parent alcohol.

Table 4
Ideal distribution requirements for orally active iron chelators, designed to treat thalassaemia

	Dist. coeff.
Good absorption from gastrointestinal tract	>0.2
Efficient liver extraction	>1.0
Poor entry into peripheral cells (Thymus, muscle, heart, bone marrow)	<0.001
Poor ability to penetrate BBB and maternal/placental barrier	<0.001

Hydroxypyridinones possessing these properties have recently been identified [20]. Thus, the hydroxyethyl-substituted compound (CP102 (2)) is not extensively metabolised and hydrophobic esters of this compound are rapidly extracted by liver. Pivoyl esters, for instance, are stable in both the lumen of the small intestine and plasma but in contrast they are rapidly hydrolysed in hepatocytes by cytoplasmic carboxyesterases (Fig. 3). By adopting this strategy it is possible to design chelators with distribution properties similar to the ideal ranges as outlined in Table 4. The concept works extremely well in mammals, the pivoyl esters leading to enhanced iron excretion in several iron overload models [21]. Unfortunately, pivalic acid possesses undesirable side effects in man when used at high doses [22], the branched acid inhibiting the carnitine cycle and thereby inducing side effects in muscle [23]. We are currently investigating alternate acids which will serve the same function of efficiently delivering hydroxypyridinones to the liver and thereby offering a realistic approach to the design of non-toxic, orally active iron chelators for the treatment of thalassaemia.

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Toxicity of iron and hydrogen peroxide: the Fenton reaction

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Abstract

Iron and hydrogen peroxide are capable of oxidizing a wide range of substrates and causing biological damage. The reaction, referred to as the Fenton reaction, is complex and capable of generating both hydroxyl radicals and higher oxidation states of the iron. The mechanism and how it is affected by different chelators, and the interpretation of results obtained in biological systems, are discussed.

Keywords: Iron mediated injury; Fenton reaction; Hydroxyl radical toxicity

1. Introduction

Iron is an essential constituent of a number of proteins involved in oxygen transport or metabolism. It must also be transported around the body, stored and made available for synthesis of iron proteins. The ability of iron to undergo cyclic oxidation and reduction is an important aspect of its function. However, such redox activity can generate free radicals and other strongly oxidizing species capable of causing a wide range of biological injury. This can occur through a variety of mechanisms. Iron can promote radical formation from physiological or xenobiotic compounds, e.g. by catalysing autoxidation, it can initiate lipid peroxidation, and react with hydrogen peroxide to produce more highly reactive and toxic species.

From the time that oxygen radicals were first implicated in biological injury, many studies have shown protection by superoxide dismutase and or catalase, and sometimes metal chelators. These effects have been widely attributed to preventing hydroxyl radical formation from the iron catalysed reaction of superoxide and hydrogen peroxide (Haber–Weiss reaction), and this reaction became the most plausible explanation for the majority of radical mediated injury and the toxicity of superoxide. However, two important properties of superoxide have recently emerged. The first is its ability to react with nitric oxide to give peroxynitrite, a strong oxidant with properties similar to the hydroxyl radical [1]. The second is the ease with which superoxide can remove iron from certain iron sulfur proteins [2]. Thus two mechanisms for superoxide toxicity independent of the Haber–Weiss reaction have been identified, the first not requiring iron or a transition metal, and the second releasing iron without requiring superoxide to act subsequently as its reductant. This means that the Haber–Weiss reaction may not have been responsible for all of the biological injury previously attribu-

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ted to it. The role of iron now needs more critical evaluation.

2. The Fenton reaction

The Haber–Weiss reaction is a specific example of the Fenton reaction. This term refers to the reaction between hydrogen peroxide and ferrous salts to produce a reactive species capable of oxidising a wide variety of organic substrates. If Fe^{2+} can be recycled from Fe^{3+}, the iron can act catalytically. This is likely to be necessary under physiological conditions, where iron availability is low. Superoxide is one potential iron reductant. Hydrogen peroxide is produced in a vast majority of biologically relevant free radical reactions. Although on its own it is not particularly toxic, there are numerous examples of damage to biological molecules, in which hydrogen peroxide and iron are implicated [3]. A large body of circumstantial evidence points toward a role of Fenton chemistry in free radical pathology.

The reaction has been studied in a large number of chemical and biochemical systems employing a variety of iron chelates and detection systems. The general finding is that most chelates cause oxidation of some but not necessarily all detectors and relative product yields for the different chelates can vary depending on the detector. Questions have therefore arisen as to whether some iron complexes are unable to produce a Fenton oxidant, whether they produce different oxidants, or whether they all produce the same oxidant that has different overall effects depending on what other secondary reactions occur. Thus, whether or not the product of the Fenton reaction is the hydroxyl radical has been a matter of contention for many years [4–9].

The simplest representation of the Fenton reaction is (1), in which there is an initial electron transfer with no bonds formed or broken in the process, and hydroxyl radicals are produced. This is termed an outer sphere mechanism.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + [H_2O_2^{\cdot -}] \rightarrow {}^{\cdot}OH + OH^- \quad (1)$$

However, on thermodynamic grounds this mechanism is extremely unfavorable and regardless of how the iron is complexed it is unlikely to occur as shown. The evidence is strongly in support of an inner sphere mechanism, forming a transient that can be regarded as a ferrous peroxide complex as in reaction (2) [6,9]. In this and subsequent reactions the iron is considered as complexed to a ligand (L) that might be EDTA, phosphate, or any biological chelator.

$$L\text{-}Fe^{2+} + H_2O_2 \rightarrow L\text{-}Fe(H_2O_2)^{2+} \quad (2)$$

$$L\text{-}Fe(H_2O_2)^{2+} \rightarrow L\text{-}Fe^{3+} + {}^{\cdot}OH + OH^- \quad (3)$$

or

$$\rightarrow L\text{-}Fe^{4+} + 2OH^- \quad (4)$$

or

$$+ R \rightarrow L\text{-}Fe^{3+} + OH^- + {}^{\cdot}ROH \quad (5)$$

$Fe(H_2O_2)^{2+}$ and Fe^{4+} are higher oxidation states of the iron that can be regarded as iron(IV) or ferryl species. The transient can break down in a variety of ways, to give the hydroxyl radical in reaction (3), or an iron(IV) species in reaction (4) or by directly oxidizing a substrate (R) in a reaction typified by reaction (5). The rates of these steps are influenced by the accessibility of the hydrogen peroxide to the iron, which in turn relates to the number of ligation sites occupied by the chelator. Thus the relative importance of reactions (3)–(5) depends on the nature of the chelator and also to some extent on the pH. Furthermore, the presence of a compound that can be directly oxidized by the transient (reaction (5)) can alter the mechanism so that little ·OH is produced. Current evidence, is consistent with this mechanism in which a reactive peroxide intermediate is formed as the initial step with all iron complexes. The way that this breaks down and whether free hydroxyl radicals are subsequently formed depends both on the chelator and on the composition of the solution.

For differences between Fenton oxidants to be functionally relevant, the oxidants must give distinguishable products or differ in their reactivities with substrates. While this would seem

straightforward to establish by comparing with ·OH generated radiolytically on the basis of product analysis or competitive kinetics, in practice it is extremely difficult. It has been done for Fe(EDTA) and Fe(nitrilotriacetate) in systems where rate constants for all the primary and potential secondary reactions are known. For Fe(EDTA), it has been shown that in most systems the Fenton product is indistinguishable from the hydroxyl radical [9]. However, there are a few substrates that can presumably react in (5) such that ·OH is no longer the predominant oxidant [10]. For Fe(nitrilotriacetate), however, ·OH is not the major product and oxidations by a ferryl species are more favored [9]. For these two similar Fe(II) chelates, therefore, differences in the relative rates of reactions (2)–(5) seem able to affect their mechanism of reaction with hydrogen peroxide. It seems likely that the behavior of iron complexed with physiological chelates is also explicable in terms of variable contributions of reactions (3)–(5).

With most Fenton detection systems, identifying the oxidant involved is not straightforward. As a general rule, the initial oxidation of the substrate generates another radical and thus sets up a sequence of radical reactions. Each step can potentially be influenced by components of the Fenton system. Spin trapping, for example with dimethylpyrroline *N*-oxide (DMPO), would appear to circumvent this problem and also have the advantage of identifying the trapped species. However, even in this case complications arise. First, DMPO-OH can be formed either directly through the addition of ·OH or by electron extraction by another oxidant such as a ferryl species followed by hydration. Also, iron complexes have the ability to oxidize or reduce the trapped radical [11]. While yields that are not as expected for ·OH can be taken as indicative of involvement of other species [8], it is only when all these secondary reactions are considered that definite conclusions can be drawn.

Another approach has been to use compounds such as salicylate that react with ·OH to give a specific ratio of 2- and 5-hydroxylated products. However, in these systems interaction of intermediates with iron chelates or other components of the reaction mixture can also distort the product ratio [12].

We have used deoxyribose oxidation extensively to investigate Fenton reaction products [13–15]. This reaction has advantages of sensitivity and simplicity, but it involves several steps and produces a number of poorly characterized products. Competitive kinetic analysis in which reactant concentrations are varied and scavengers added have provided useful mechanistic information, but the potential for reaction at different steps in the sequence imposes limitations. Our results with this system are compatible with Fe^{2+}(EDTA) reacting with hydrogen peroxide to produce ·OH. With Fe^{2+} in phosphate buffer, our results are not consistent with a reaction due solely to free ·OH or site localized ·OH, and we had to conclude that the system was too complex for definitive identification of the Fenton oxidant [15].

Cells or whole organisms are even more problematic. Radical reactions, whether or not they involve Fenton chemistry, involve a series of steps and intermediates. In cells these are compounded with large concentration gradients and numerous potential intermolecular associations, poorly characterised iron species and a requirement for an unrealistically high concentration of a scavenger to inhibit reactions of a species such as ·OH efficiently. Therefore, appropriate scavengers and chelators can be used as probes for Fenton chemistry in vivo, but as concluded by Goldstein et al. [9], to decide whether reactions are due to hydroxyl radicals or a higher oxidation state metal is an impossible task.

3. Reductants of catalytic iron

Many ferrous chelates react with hydrogen peroxide to produce an oxidizing species, but for the iron to act catalytically, the ferric chelate must be reducible. Chelators affect the reduction potential of iron, and therefore its effectiveness as a catalyst. Thus, particular reductants promote Fenton chemistry with some iron chelates but not others. Superoxide, which is a mild reductant, functions efficiently with Fe(EDTA). However, other chelates, e.g. with citrate, ATP and ADP,

are much less effective catalysts and Fe(diethylenetriamine-penta-acetic acid) is essentially inactive in the superoxide-driven Fenton reaction [16]. With more strongly reducing radicals such as derived from paraquat or adriamycin, all these chelates are good catalysts of Fenton chemistry [13]. Reductants like ascorbate fall within these two extremes.

4. Prevention of Fenton reactions

The ideal chelator for preventing Fenton chemistry must stabilize iron in a redox state that is inert either to oxidation by hydrogen peroxide or to reduction by commonly encountered reducing agents. The iron transport proteins, transferrin and lactoferrin fall into the latter category. Desferrioxamine largely meets this criterion, although strong reductants like the paraquat radical can reduce the ferric complex. 1,10-Phenanthroline and bipyridine, on the other hand, prevent the reaction of Fe^{2+} with hydrogen peroxide. It is desirable to use both types of chelator when establishing whether a Fenton-type reaction is involved in biological damage. However, it is important to keep in mind that these compounds can bind other metals, and desferrioxamine has poor penetrability for many cells. It also scavenges a wide range of oxidant species [17,18] so its effects are not always due to chelation.

5. Site specific reactions

One effect of chelators is to remove iron bound to other molecules and thereby prevent it from undergoing site localized reactions with them. There is good evidence that bound iron can cause site specific damage to proteins and DNA [19,20]. Chelators can prevent such reactions even if they do not inhibit Fenton chemistry. Probably the mechanism is best represented by reaction (5), with R localized close to the iron binding site, rather than a reaction of ·OH, although this is not easily distinguished experimentally. There has been a tendency to attribute any reactions requiring hydrogen peroxide that are influenced by chelators and do not exhibit the expected competition kinetics for a reaction of free ·OH to a site specific reaction of ·OH with the detector [21]. In many cases this is without evidence of metal binding. However, as described above, chelators can also influence the mechanism of the Fenton reaction by modifying the redox potential of iron and the ease with which it can form a transient complex with hydrogen peroxide. In view of these multiple effects on the reactivity of iron, site specificity is not the only interpretation of apparently anomalous results. Evidence that the metal binds to the target should be demonstrated before site specificity is invoked.

6. Physiological Fenton catalysts

Regarding the physiological forms of iron that can participate in Fenton reactions, it is widely accepted that small amounts of low molecular weight or loosely bound iron exist intracellularly, in transit between different protein pools. This iron is poorly characterized. Its existence is supported, however, by the control it appears to exert on iron homeostasis through the iron responsive element binding protein [22]. This is a cytosolic iron-sulfur protein, aconitase, that acts as an iron sensor. When iron levels are low it loses one of its irons, and its affinity for the iron responsive element in mRNA is increased. This enhances translation of the transferrin receptor and downregulates translation of ferritin and other proteins involved in iron utilization and generally places the cell in a position for acquiring more iron for heme synthesis. As iron levels increase, an extra iron becomes incorporated into the binding protein, its affinity for the mRNA is decreased and effects on translation are reversed.

The bulk of cell iron is present in ferritin and heme proteins. Iron within ferritin does not participate in redox reactions, but it can be reductively released by a variety of radicals [23]. Superoxide is one of these. However, it and other cellular reductants are relatively inefficient and ferritin may normally be more of a protector than contributor to cell injury. More reducing radicals, such as the paraquat and adriamycin

radicals, and strong reductants such as 6-hydroxydopamine, are much more effective at releasing ferritin iron, and this could contribute to their cytotoxicity.

Heme proteins react with hydrogen peroxide but do not release detectable ·OH [24]. Compound II complexes of peroxidases and the equivalent forms of hemoglobin and myoglobin are, however, well characterized ferryl species. The reaction of hemoglobin or myoglobin with hydrogen peroxide gives a ferryl radical, in which the radical character is localized on the globin. These complexes are strongly oxidizing as demonstrated by their oxidation of numerous peroxidase substrates [25] and promotion of processes such as lipid peroxidation. Therefore, while not true Fenton reagents, hemoglobin, myoglobin and other heme proteins must be regarded as potential candidates for iron mediated injury by hydrogen peroxide.

Although hydroxyl radicals, and Fenton systems, are capable of initiating lipid peroxidation, in most biological systems this does not appear to be the prime mechanism that operates. Catalase is seldom protective, and reactions of iron with even small amounts of lipid hydroperoxides to give peroxyl and alkoxyl radicals are more likely to propagate peroxidation.

7. Conclusion

The majority of ferrous chelates react with hydrogen peroxide to produce a Fenton oxidant. On thermodynamic grounds the reaction almost certainly occurs by an inner sphere mechanism to produce initially a peroxide-bound transient. There is experimental evidence to indicate that this transient can react with oxidizable substrates either as a ferryl species or break down to give hydroxyl radicals. The relative importance of the different oxidation mechanisms depends on how the iron is chelated and on what substrates are present. While these effects can be distinguished in a chemical system, in cells or whole organisms this may be impossible. However, provided the potential for broad reactivity is accepted and atypical behavior for ·OH is not automatically assumed to be due to a particular mechanism such as site specificity, then in terms of biological relevance the exact nature of the Fenton oxidant is probably not critical. Regardless of its identity, this oxidant will react with a multitude of biological constituents and show little discrimination. This means that molar concentrations of any one scavenger would be necessary to trap it effectively in a cell or biological fluid. Hydroxyl radical scavenging ability of pharmacological agents, therefore, is largely irrelevant. If such agents are effective as antioxidants, they are much more likely to be scavenging other less indiscriminate radicals or acting by an alternative mechanism.

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Chemical structure – teratogenicity relationships, toxicokinetics and metabolism in risk assessment of retinoids

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Abstract

Retinoic acid, an oxidative metabolite of vitamin A, is involved in the control of many biological processes including embryonic development and excess as well as deficiency of retinoids has been found to be teratogenic. The effects of retinoids in normal as well as abnormal development may be mediated by two members of retinoid receptors, the RARs and RXRs, which exhibit specific temporal and spatial expression during development. Evidence accumulates that any alteration of this complex retinoid system may be related to teratogenic effects. Here we investigate the influence of toxicokinetic parameters, including aspects of metabolism and placental transfer, on the teratogenic potency of retinoids. It is demonstrated that activation (oxidation of retinoic acids; hydrolysis of glycoconjugates) and deactivation reactions (isomerization from *trans*- into *cis*-configuration; β-glucuronidation) relate to teratogenesis. The β-glucuronides of retinoic acids show poor placental transfer and prolonged presence in the maternal organism. Non-retinoid compounds such as antiepileptic agents may exert some of their teratogenicity via alteration of endogenous retinoid levels.

Keywords: Vitamin A; Retinoic acid; Teratogenesis; Retinoyl-β-glucuronides; Retinoid receptor ligands; Placental transfer

1. Introduction

Retinoic acid, an oxidative metabolite of vitamin A, plays a key role in many biological processes including growth and differentiation of epithelial tissues, spermatogenesis, vision and embryonic development [1,2]. This extremely broad range of effects is thought to be mediated by nuclear retinoid receptors as well as cytosolic retinoid binding proteins [3]. There are two groups of retinoid receptors: RARα, RARβ and RARγ as well as RXRα, RXRβ and RXRγ [4]. These receptors belong to the steroid/thyroid hormone receptor superfamily and function as ligand-activated transcription factors controlling the expression of numerous responsive genes. All-*trans*-retinoic acid acts as ligand for the RAR-receptors, while 9-*cis*-retinoic acid can bind to both the RAR- and RXR-receptors. The specific expression of these receptors (and their isoforms), as well as the concentrations of the

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Abbreviations: AUC, area under the concentration-time curve; C_{max}, maximal concentration; CRBP, CRABP, cellular retinol and retinoic acid binding protein, respectively; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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retinoic acid ligands may be crucial factors in the control of the transcription of responsive genes.

Vitamin A excess and deficiency, as well as the administration of low doses of retinoic acid have been shown to result in teratogenic effects [5–8]. All-*trans*-retinoic acid is present endogenously in the embryo and shows a specific distribution within the embryo. The RAR and RXR families of retinoic acid receptors also show a very distinct spatial as well as temporal distribution within the developing embryo. In addition, the cytosolic retinol binding proteins (CRBPs) as well as the retinoic acid binding proteins (CRABPs) also exhibit a very specific expression pattern in the embryo, which could also be crucial for the action for endogenous and exogenous retinoids [9]. These proteins may be involved in the metabolism of retinol and retinoic acid as well as in the control of the 'free' concentration of all-*trans*-retinoic acid, which is available for nuclear receptor binding. Thus, the multiplicity of the receptors (RARs and RXRs and their isoforms), the formation of homo- and hetero-receptor dimers, the two natural ligands (all-*trans*- and 9-*cis*-retinoic acids), the multiplicity of cytosolic retinoid binding proteins (CRABPs and CRBPs) and the very specific temporal and spatial expression of retinoid receptors, binding proteins and ligands within the embryo could enable an extremely wide range of signaling pathways that control normal development.

We have therefore investigated toxicokinetic and metabolic parameters in maternal plasma and embryo and related these patterns to the teratogenic activity of the administered retinoid compounds [6–8]. We have also demonstrated that antiepileptic agents can alter plasma concentrations of retinol and several retinoic acids, and these effects may be involved in the mechanism of anticonvulsant teratogenesis [10].

2. Results and discussion

2.1. Placental transfer of retinoids

The chemical structure of retinoids has a major influence on placental transfer. All-*trans*-retinol and all-*trans*-retinoic acid show extensive placental transfer, while the *cis*-isomers of retinoic acid (9-*cis*, 13-*cis*, 9,13-di-*cis*-retinoic acid) show a much more limited placental transfer in the mouse, rat and rabbit (Table 1). The reasons behind these structure-specific differences are not clear, and may reside in the differential binding affinities of these retinoic acids to protein(s) which may possibly exist for their specific transport into the embryonic compartment. Interestingly, the placental transfer of 13-*cis*-retinoic acid was much greater in the monkey than in the rodent species, and the differing placental structures between the rodents (yolk sac placenta) and the monkey (chorioallantoic placenta) were suggested to be one reason for these interesting and potentially important differences.

2.2. Plasma clearance and metabolism

The 13-*cis*-retinoic acid is a potent teratogen in monkeys, and even more potent in the human, and extensive transfer to the monkey embryo may be important in this aspect. Also the primary metabolic pathway proceeds to the 13-*cis*-4-oxo-retinoic acid in the monkey, which may serve as an activation pathway of teratogenesis. In contrast, in rats and mice the main plasma metabolite is the 13-*cis*-retinoyl-β-glucuronide which shows very poor placental transfer and may have little intrinsic teratogenic activity. Furthermore, the plasma clearance of 13-*cis*-retinoic acid is much greater in rats and mice than in monkeys. The low teratogenic potency of 13-*cis*-

Table 1
Placental transfer of retinoids (mouse, rat, rabbit)

Retinoid	Embryo/maternal plasma ratio
RAG	
all-*trans*	0.01–0.03
13-*cis*	0.01–0.03
9-*cis*	0.01–0.03
RA	
9,13-di-*cis*	0.01–0.03
13-*cis*	0.05–0.1
9-*cis*	0.15
all-*trans*	0.5–2

Data from Refs. [11–16].

Table 2
Species variation of 13-*cis*-retinoic acid teratogenesis

Parameter	Mouse/rat	Rabbit	Monkey/human
Clearance	Fast	Slow	Slow
AUC	Low	High	High
Placental transfer	<0.1	<0.1	0.4 (monkey)
Maternal metabolism	Deactivation to β-glucuronides	Activation to 4-oxo-met.	

Data from Refs. [11,12,14–17,19,20].

retinoic acid in the rat and mouse may therefore be explained by three factors (Table 2): limited placental transfer, rapid plasma clearance and extensive metabolic detoxification; on the other hand, the high teratogenic activity of this retinoid in the monkey (and possibly the human as well) is the result of more extensive placental transfer, slower plasma clearance and extensive metabolism to the active 4-oxo-metabolite (Table 2).

2.3. Alteration of endogenous retinoid levels as possible mechanism of teratogenesis of antiepileptic agents

The major antiepileptic drugs used for the control of seizures can induce developmental toxicity when administered during pregnancy. Vitamin A and retinoids are thought to control many processes of embryonic development including growth, differentiation and morphogenesis. We have tested the hypothesis that the teratogenic action of antiepileptic agents is mediated via alteration of the endogenous vitamin A – retinoid metabolism. Retinol and its oxidative metabolites, all-*trans*-, 13-*cis*- and 13-*cis*-4-oxo-retinoic acid were measured in the plasma of 75 infants and children treated with various antiepileptic drugs for the control of seizures, and in 29 untreated controls of comparable age [10]. Retinol levels increased with age, while the concentrations of retinoic acid compounds did not exhibit age-dependency. Valproic acid monotherapy increased retinol levels in the young age group and a trend toward increased retinol concentrations was also observed in all other patient groups (Table 3). The levels of the oxidative metabolites 13-*cis*- and 13-*cis*-4-oxo-retinoic acids were strongly decreased in all patient groups treated with phenytoin, phenobarbital, carbamazepine and ethosuximide, in combination with valproic acid, to levels which were 27% and 6% of corresponding control values, respectively (Table 4). Little change was observed with all-*trans*-retinoic acid except in one patient group treated with valproic acid – ethosuximide cotherapy where increased levels of this retinoid were found. Our study indicates that therapy with antiepileptic agents can have a

Table 3
Plasma concentrations of retinol in infants and children treated with antiepileptic drugs

Group	Age group: retinol plasma conc. (ng/ml; means ±S.D.)		
	0–6 years	>6 years	All subjects
Controls	232 ± 36 ($n = 15$)	351 ± 62 ($n = 12$)	285 ± 77 ($n = 27$)
VPA	312 ± 73 ($n = 10$)[a]	379 ± 102 ($n = 27$)	363 ± 99 ($n = 39$)[b]
VPA + PT	–[e]	393 ± 116 ($n = 4$)	393 ± 116 ($n = 4$)[a]
VPA + CM	272/317 ($n = 2$)[b]	436/544 ($n = 2$)[b]	392 ± 123 ($n = 4$)[b]
VPA + PB	357 ± 40 ($n = 3$)[d]	414 ± 39 ($n = 4$)	389 ± 47 ($n = 7$)[a]
VPA + ES	–[e]	431 ± 137 ($n = 6$)	405 ± 143 ($n = 7$)[a]
VPA + combination	360 ± 20 ($n = 3$)[d]	420 ± 77.2 ($n = 6$)	399 ± 69 ($n = 9$)[d]

Abbreviations: CM, carbamazepine; ES, ethosuximide; PB, phenobarbital; PT, phenytoin; VPA, valproic acid.
Data from Ref. [10]
Significantly higher than in controls (Student's *t*-test): [a] $p < 0.005$; [b] $p < 0.05$ [c] $p < 0.01$; [d] $p < 0.0005$; [e] insufficient number of patients.

Table 4
Plasma concentrations of polar retinoids in infants and children treated with antiepileptic drugs

Group	Plasma conc. of retinoic acids (RA) (ng/ml; means ± S.D.)			
	n	all-*trans*-RA	13-*cis*-RA	13-*cis*-4-oxo-RA
Controls	29	1.07 ± 0.26	0.97 ± 0.31	2.26 ± 0.93
VPA	40	1.13 ± 0.39	1.19 ± 0.89	2.22 ± 1.82
VPA + PT	5	1.07 ± 0.36	0.30 ± 0.19[a]	0.17 ± 0.38[a]
VPA + CM	4	1.17 ± 0.12	0.34 ± 0.11[b]	0.22 ± 0.44[b]
VPA + PB	7	0.92 ± 0.22	0.35 ± 0.26[a]	0.28 ± 0.48[a]
VPA + ES	8	1.35 ± 0.23[c]	0.58 ± 0.27[c]	1.08 ± 0.71[c]
VPA in comb.[d]	11	0.91 ± 0.31	0.23 ± 0.31[a]	0.14 ± 0.48[a]

n, number of subjects. For abbreviations of antiepileptic drugs see Table 3.
Data from Ref. [10]
Significantly different from corresponding controls (Student's *t*-test): [a] $p < 0.0001$; [b] $p < 0.001$; [c] $p < 0.01$; [d] Patients treated with VPA and two or three additional antiepileptic drugs.

significant effect on endogenous retinoid metabolism. Because of the importance of retinoids for signaling crucial biological events during embryonic development, altered retinoid metabolism may be an important factor in antiepileptic drug teratogenesis. It will be important to study how such altered plasma retinoid concentrations will affect tissue retinoid levels, particularly in those areas most susceptible to retinoid excess and deficiency. The biological consequences of altered tissue retinoid concentrations could be wide-spread.

Although all-*trans*-retinoic acid, considered to be the most active endogenous retinoid, was not greatly altered in our present study, the effects of anticonvulsants on retinol, 13-*cis*-retinoic acid and its 4-oxo-metabolite are likely to be of great importance.

The coadministration of phenobarbital also drastically decreased plasma levels of 13-*cis*-retinoic acid and its 4-oxo-metabolite [10]. Phenytoin and carbamazepine may have acted similarly to phenobarbital in the reduction of endogenous retinoids. It is interesting to speculate why the levels of all-*trans*-retinoic acid were not significantly altered in these patients by administration of the inducing antiepileptic agents. It may be that the coadministered valproic acid, previously shown to be an inhibitor of several enzymatic reactions, may have offset the inducing activity of the other anticonvulsants; this hypothesis is presently under investigation.

2.4. Pharmacokinetic parameters: AUC vs. C_{max}

We have demonstrated that the AUC-values (area under the concentration-time curve values) of active retinoids attained in the embryonic compartment during sensitive stages of gestation are appropriately correlated with observed teratogenic activity [6,7,20]. Using AUC-values, a rational species comparison is possible and experimental results may be extrapolated to human exposures. It has been shown that the teratogenic effects of other drugs such as caffeine [21] and valproic acid [22] can be related to the maximal concentrations (C_{max})-values rather than the AUCs. It is even possible that one particular effect (digit malformation) induced by a drug such as methoxyacetic acid can be correlated with the AUC-values, while another effect (exencephaly) induced by the same drug can be correlated with C_{max}-values [23]. It may be inferred from that study that the crucial toxicokinetic parameter will depend not only on a particular drug, but also on a particular organ system and developmental period.

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INFORMATION ON INFORMATION

The introduction of Elsevier Electronic Subscriptions (EES) represents an important step in Elsevier Science's commitment to assembling electronic primary information for the digital library. Following the experimental TULIP Project and the commercial availability of CAPCAS, EES is the next in a series of steps toward providing information for electronic libraries.

Elsevier Science is committed to working with customers in a highly interactive mode to define products and services for the electronic library and move journals to the desktop. Projects that have been initiated in the past, like TULIP and CAPCAS, have evolved around delivering information in open standards format to enable customers to provide for:

- *flexibility in the way information is presented and used;*
- *safety of investments with regard to electronic infrastructure;*
- *possibilities for shaping solutions interactively with users.*

AN IMPORTANT STEP FORWARD...

Launched in early 1995 on a pilot basis with a limited number of institutions, EES is a licensing program which offers electronic versions of the entire range of Elsevier's paper journals — over 1100 journal titles totaling more than 1.5 million journal pages annually. The sheer volume available from Elsevier Science in electronic format is unprecedented in the scientific publishing industry.

EES enables customers to start developing a baseline system which provides sufficient functionality and content to form the foundation of a truly electronic library. This program represents an important step in the logical transformation of the fundamental ways in which information is acquired, used and shared within the scientific research community.

The major benefits of the EES program are the possibilities and level of flexibility it offers. Requirements can be defined or redefined through the program implementation and products adapted accordingly to better serve the requirements of end-users, information specialists and librarians.

With EES the library can overcome many of the inherent restrictions of a paper-based environment. At a basic level, added functionalities include multiuser simultaneous access and access from the end-user's desktop. Depending upon the sophistication of the local implementation, more functionality can be included: browsing and searching using navigational and retrieval tools, various alerting functions, profiling and remote access. Librarians can also monitor journal usage easily and accurately.

AVAILABILITY...

This new subscription service, initially available on a pilot program basis, offers libraries electronic versions of Elsevier Science's traditional research and professional journals. The electronic editions are available either in addition to or in lieu of paper journals. Journals under the imprints of Elsevier, Pergamon, North-Holland and Butterworth-Heinemann are available under EES and will include not only full-length scientific articles but all editorial material including product reviews, correspondence, editorial notes, etc.

Flexible pricing/licensing models are geared to aid the efficient dissemination of scientific, technical and medical information.

Initially EES is delivered to pilot program participants on magnetic tape or CD-ROM, either weekly or bi-weekly depending upon a customized journal list profile of the customer.

EES IN THE LIBRARIES...

EES is intended to be implemented with either a library's own or third-party software. For example, librarians may choose OCLC's SiteSearch® system, which allows building, maintaining and searching databases locally, and OCLC's Guidon® graphical user interface. Elsevier Science is also in negotiation with other technology providers to be able to offer users a broad scope of possibilities to implement EES, based on individual needs.

The technology basis for EES is a genuinely open architecture, in which content adheres to open standards making it inherently possible to use the information in any system that also adheres to these open standards. In this way libraries have the flexibility to build their electronic journal infrastructure on the basis of this service and then extend and modify it in the future.

The information technology requirements at the institutional and end-user levels, however, are not trivial for organizations interested in implementing EES. Sites must be well equipped to deal with the technical and organizational aspects of a large scale image-based information service. Technical issues include bandwidth and storage requirements intrinsic to the page image format. A complex set of issues and choices concerning the design and implementation of the hardware and software infrastructures must be addressed. Organizational commitment to support user training and feedback is essential, especially during the early stages, to increase the speed of implementation and satisfaction with the end result.

ABOUT THE FILES ...

The electronic journals are provided in cover-to-cover bitmapped images — black/white single-page TIFF files with a resolution of 300 dpi, compressed using the CCITT Fax Group IV encoding scheme. Bibliographic header information, including the abstract and keywords, when present, are provided in structured SGML-tagged text. The full text is provided as an unedited and unstruc-

ELSEVIER ELECTRONIC SUBSCRIPTIONS



tured ASCII file created by applying an OCR (optical character recognition) process on the page image. This format is similar to the one used in TULIP, the experiment in full-text electronic journals undertaken by Elsevier Science and nine major research universities that began in 1991.

Collections of journals are delivered in datasets, each containing one master index file and the Dataset.toc file. These include complete bibliographic information as well as all relevant cross reference data (e.g., relationships between page images and specific articles and the cluster of articles published in a journal issue).

END-USER ACCESS ...

Depending upon the implementation, page images can be displayed on the end-user's computer display and locally available using image viewer software with capabilities such as zooming, panning and paging. Page images can also be printed on most standard laser printers, allowing for printing at the user's office as well as on high-volume production laser printers located centrally in the library or computer center.

Information can be retrieved using three different basic approaches:

- Searching all or any combination of structured bibliographic data fields and/or the full-text ("raw" ASCII files).
- Browsing such features as tables of contents, journal cover representations, statements of aims and scope, and additional classification features for each journal title.
- Profiling based on the individual users' predefined interest areas to automatically notify users by e-mail about new articles of potential interest.

LICENSING AND PRICING...

Licensing agreements and the associated pricing models are based on the level of implementation and functionality desired by each customer organization.

Licensing facilitates wider access to the information enhancing flexibility and greater functionality. EES licenses are available to institutions with single or multiple sites.

The entire user community, as defined within the license, may access EES. Users will be entitled to search and display the electronic files and make prints and download without limitations. Licensed institutions are not authorized to distribute files to anyone outside the defined categories of users covered by the license.

Customized pricing is based on the details of the licensing agreement. It is possible to take a license option that entitles the users to perform all the functions as stated above (search, browse, display, download, print). Alternately, there exists the option of paying a lower subscription fee, plus a charge each time an article is downloaded or printed.

EES AND OTHER ELSEVIER ELECTRONIC PROJECTS...

EES is the broadest of several Elsevier Science projects based on the implementation of bitmapped page images and SGML-tagged bibliographic header information. Most notable among these are TULIP and CAPCAS, and EES builds upon the expertise gained through both of these endeavors. The technology used in EES is consistent with that being employed in a number of electronic full-text delivery endeavors, on both a commercial and test-pilot basis, currently offered by leaders in the scientific publishing industry.

At the same time, Elsevier Science is installing systems to ensure the possibility of delivering full text SGML-tagged and PDF or PostScript files on a broad scale in the future. Several projects already available use this technology to electronically deliver single journals in specific scientific areas. Further information about these programs is available from the contact name listed below.

IN PARTNERSHIP WITH LIBRARIES...

Elsevier Science is building the EES service in close partnership with the library community, with the first year's implementation being very much an evolutionary period. In light of the diversity of requirements and the involvement of Elsevier personnel in getting successful implementation in place, customization is necessary and this is currently possible with a limited number of participants.

Elsevier Science will work with these interested institutions to define the technical and organizational infrastructure needed to support a successful implementation. The choices to be made as to the functionalities required and resolving technical challenges are critical to the success of each site's implementation. By working closely with program participants to refine technical standards, service requirements and price schedules, the end result will be the creation of a service tailored to effectively address users' needs. Wider availability is anticipated in 1996.

FOR FURTHER INFORMATION...

Elsevier Science will provide you with further information about EES or place you on a mailing list to receive updated information on new EES and electronic product developments. Please contact:

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