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The Center for Environmental Medicine has been established at the Medical College of Ohio, Toledo, Ohio with Dr Michael A. Pereira, Ph.D. as Director. The Center consists of two division, Molecular Toxicology Division and Respiratory Toxicology Division. The Molecular Toxicology Division has initiated studies to better understand the toxicity and carcinogenic activity of compounds of interest to the U.S. Air Force. These studies have emphasized trichloroethylene, a major contaminant around Air Force bases. Two major metabolites of trichloroethylene, dichloroacetic acid and trichloroacetic acid were also evaluated, since they are believed to be its active metabolites and are used in the hazard and risk assessment of trichloroethylene by the U.S. Air Force and EPA. A letter of agreement with Wright-Patterson AFB (WPAFB) was formalized to allow their collaboration. This has allowed tissue specimens to be sent between WPAFB and the Medical College of Ohio including the immunohistochemical evaluation by an Air Force pathologist. Two Air Force personnel were trained at the Medical College of Ohio in the technique of measuring trichloroethylene increase expression of mRNA for proto-oncogenes and a graduate student at the Medical College spent the summer of 1995 at WPAFB being trained by their pathologist in immunohistochemical techniques.					
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CENTER FOR ENVIRONMENTAL MEDICINE

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February 28, 1996

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SUMMARY

The Center for Environmental Medicine has been established at the Medical College of Ohio, Toledo, Ohio with Dr. Michael A. Pereira, Ph.D. as Director. The Center consists of two divisions, Molecular Toxicology Division and Respiratory Toxicology Division. The Molecular Toxicology Division has initiated studies to better understand the toxicity and carcinogenic activity of compounds of interest to the U.S. Air Force. These studies have emphasized trichloroethylene, a major contaminant around Air Force bases. Two major metabolites of trichloroethylene, dichloroacetic acid and trichloroacetic acid were also evaluated, since they are believed to be its active metabolites and are used in the hazard and risk assessment of trichloroethylene by the U. S. Air Force and EPA. A letter of agreement with Wright-Patterson Air Force Base (WPAFB) was formalized to allow their collaboration. This has allowed tissue specimens to be sent between WPAFB and the Medical College of Ohio including the immunohistochemical evaluation by an Air Force pathologist. Two Air Force personnel were trained at the Medical College of Ohio in the technique of measuring trichloroethylene increase expression of mRNA for proto-oncogenes and a graduate student at the Medical College spent the summer of 1995 at WPAFB being trained by their pathologist in immunohistochemical techniques.

This collaboration included studies that 1) evaluated the tumor promoting activity and mechanism of trichloroethylene, dichloroacetic acid and trichloroacetic acid in mouse liver; 2) and 2) determined the biochemical and molecular activity of trichloroethylene in mouse liver. The Respiratory Toxicology Division has initiated the following studies: Program information has been obtained to determine the appropriate site, space needs, design criteria and project cost for a building to house the Center for Environmental Medicine.

The Center for Environmental Medicine at the Medical College of Ohio was established by the Board of Trustees on March 25, 1991. Dr. Michael A. Pereira was recruited to be Director of the Center for Environmental Medicine as well as Director of the Division of Molecular Toxicology. Dr. Dan E. Olson joined the Center for Environmental Medicine as Director of Respiratory Toxicology. Ms. Marilyn Cline was appointed Administrative Assistant/Secretary for the Center. The following personnel are members of the Center:

Molecular Toxicology Division

Michael A. Pereira, Ph.D.	MCO
William T. Gunning, III, Ph.D.	MCO
James A. Hampton, Ph.D.	MCO

Respiratory Toxicology Division

James C. Willey, M.D.	MCO
Dan E. Olson, M.D., Ph.D.	MCO
Gourie Shanker, Ph.D.	MCO, University of Toledo
Raj Sawhney, Ph.D.	MCO
Ken W. Cairns, M.S.	MCO
Erin Coy, M.S.	MCO
Patricia Metting, Ph.D.	MCO
Beverly Giammara, M.S.	MCO

Although the Center for Environmental Medicine is divided into two divisions, collaborative research is encouraged between them.

MOLECULAR TOXICOLOGY DIVISION

In order to ensure that the work performed by the Center for Environmental Medicine under this grant with the U.S. Air Force is pertinent to the mission of the Air Force, Dr. Michael A. Pereira has met eight times with the scientists at Wright-Patterson Air Force Base, Toxic Hazards Division, Armstrong Aerospace Medical Research Laboratory and presented a summary of this Grant at AFOSR Project Review. A letter of agreement with Wright-Patterson Air Force Base (WPAFB) was formalized to allow their collaboration. This has allowed tissue specimens to be sent between WPAFB and the Medical College of Ohio including the immunohistochemical evaluation by an Air Force pathologist. Two Air Force personnel were trained at the Medical College of Ohio in the technique of measuring trichloroethylene increase expression of mRNA for proto-oncogenes and a graduate student at the Medical College spent the summer of 1995 at WPAFB being trained by their pathologist in immunohistochemical techniques.

Wright-Patterson Air Force Base is establishing in their laboratory procedures for the immunohistochemical detection of growth factors and proto-oncogenes and in situ hybridization

for determination of their expression. Dr. Pereira sent to Wright-Patterson Air Force Base tissue from the animals exposed to trichloroethylene, dichloroacetic acid and trichloroacetic acid as part of the studies performed under this Grant. These specimens were used as controls in the development and validation of immunohistochemical procedures in the Air Force laboratory.

Carcinogenicity and Tumor Promoting Activity of Trichloroethylene in B6C3F1 Mice

Trichloroethylene (TCE) is an organic solvent used widely by industry and the U.S. Air Force as a metal degreaser and cold cleaner of fabricated metal parts. Although production has decreased over the last 25 years, it is still approximately 120 million pounds per year in the United States. This widespread use of trichloroethylene has resulted in multimedia environmental pollution with measurable quantities found in and near surface water, ground water, and soil as well as in high percentage of superfund sites. Contamination at and around bases has resulted in the U.S. Air Force spending millions of dollars a year in the control and clean-up of trichloroethylene.

In mice, trichloroethylene has been demonstrated to induce both liver and lung tumors and in rats, males had an excess of kidney tumors. This has resulted in the U.S. EPA proposing to classify trichloroethylene as a category B2 (probable human carcinogen). However, the Science Advisory Board of the U.S. EPA has stated that the evidence for the carcinogenicity of trichloroethylene falls rather on a continuum between category B2 and category C (possible human carcinogen). Recently, the American Conference of Governmental and Industrial Hygienists (ACGIH) has established the classification of trichloroethylene as a group A5 substance, not suspected as a human carcinogen. The ACGIH classification of trichloroethylene relied heavily upon the major epidemiological study performed on the cohort of over 14,000 workers at the Hill Air Force Base in Utah. The wide range in the classification of the carcinogenic hazard of trichloroethylene to humans is mainly due to differing opinions as to the applicability of the carcinogenic activity observed in mouse liver and in male rat kidney to estimate the potential carcinogenic hazard to humans. Under this grant, we have initiated a study to determine the mechanism and dose response relationship of the carcinogenic activity of trichloroethylene in mouse liver. The mechanistic studies will attempt to determine the extent to which the carcinogenic activity demonstrated in mouse liver is applicable to predicting the carcinogenic activity in humans and the dose response relationship studies will supply quantitative data to improve any risk assessment of trichloroethylene.

Dichloroacetic acid (DCA) and trichloroacetic acid (TCA) are two major metabolites of trichloroethylene and for which there is a lot of information about their carcinogenic activity and pharmacokinetics. Therefore, they have been used as a surrogate in the risk assessment of the carcinogenic activity of trichloroethylene. The concentration-response relationships for the hepatocarcinogenic activity of DCA and TCA were determined in female B6C3F1 mice. Dichloroacetic acid or trichloroacetic acid at 2.0, 6.67 or 20.0 mmol/l, were administered to the mice in the drinking water starting at seven to eight weeks of age and until sacrifice after 360 or 576 days of exposure. The relationships of the yield of foci of altered hepatocytes, hepatocellular adenomas and hepatocellular carcinomas to the concentration of DCA and TCA in the water, was best described by second order and linear regressions, respectively. The liver-to-body

weight ratio increased linearly for both DCA and TCA, as did the vacuolization of the liver induced by DCA. The foci of altered hepatocytes and tumors in the animals treated with DCA were predominantly eosinophilic and contained glutathione S-transferase- π (GST- π , over 80% of the lesions), while the tumors induced by TCA were predominantly basophilic and lacked GST- π , including all eleven hepatocellular carcinomas. Therefore, the carcinogenic activity of DCA and TCA appeared to differ both with respect to their dose-response relationship and to the characteristics of precancerous lesions and tumors.

The hepatic tumor promoting activity for dichloroacetic acid (DCA) and trichloroacetic acid (TCA) was also determined in female B6C3F1 mice initiated on day 15 of age with 25 mg/kg *N*-methyl-*N*-nitrosourea (MNU). The mice were administered the chloroacetic acids in the drinking water starting at seven weeks of age and continuing until sacrificed 31 or 52 weeks later. Both chloroacetic acids promoted MNU-initiated foci and tumors, however their concentration-response relationships differed being exponential and linear for DCA or TCA, respectively. Lesions promoted by DCA but not TCA, regressed upon termination of exposure at 31 weeks. Foci and tumors promoted by DCA were eosinophilic and contained glutathione S-transferase- π (GST- π), while TCA promoted basophilic tumors lacking GST- π . Hence, tumor promotion similar to the carcinogenic activity of DCA and TCA differed both with respect to their concentration-response relationships and to the characteristics of precancerous lesions and tumors.

In collaboration with Wright-Paterson AFB, the lesions induced and promoted by DCA and TCA were further characterized and those of trichloroethylene will continue to be characterized. The lesions induced by DCA were positive for the growth factor TGF- α and the immediate-early proto-oncogenes *c-myc* and *c-jun* while those induced by TCA were only positive for *c-jun* (Table 1). Trichloroethylene has been reported to induce tumors (4/20, 20%) which have lost at least part of chromosome 6. This loss of heterozygosity (LOH) would suggest the loss of a tumor suppressor gene. We therefore determined the extent to which tumors induced by DCA and TCA also lost this chromosome. A similar percentage (28.6%) of TCA but not DCA induced tumors, lost at least part of this chromosome. Similar to trichloroethylene, TCA treatment resulted in the loss of a large portion of the chromosome, however unlike trichloroethylene, the C57BL/6 and not the C3H/He chromosome was lost.

Some animals that were initiated with MNU or DMBA but not subsequently treated with any of the three test agents, as well as animals that were not initiated with either carcinogen but received the high dose of TCA, were sacrificed at 537 days of age. The longer duration was required to obtain tumors large enough (>5.0 mm) to observe and harvest at necropsy. These tumors will be analyzed for the mutation spectrum in the activated *ras* oncogene family in order to determine the mutation spectrum for tumors induced in mouse liver by MNU, DMBA and TCA. Tumors obtained from animals that were initiated with MNU or DMBA and then promoted with one of the three test agents, will be evaluated to determine whether they contain the mutation spectrum distinctive of the initiating chemical. This would demonstrate whether trichloroethylene and its two metabolites promoted the occurrence of tumors derived from cells in which MNU/DMBA induced mutations in the *ras* oncogene family.

Table 1. Characterization of DCA and TCA-Induced Lesions

Characteristic	DCA		TCA	
	Non-Involved	Lesions	Non-Involved	Lesions
1. H&E	Normal	Eosinophilic	Normal	Basophilic
2. GST- π	Negative	Positive	Negative	Negative
3. Glycogen	Accumulation	Normal	Normal	Normal
4. Lost part of Chromosome 6	NA	0/14	NA	6/21 (28.6%)
4. TGF- α	Negative	Positive	Positive	Negative/Positive
5. TGF- β	Positive	Negative	Negative/Positive	Negative
6. c-Myc	Negative	Positive	Negative/Positive	Negative
7. c-Jun	Negative	Positive	Negative	Positive
8. c-Fos	Negative	Negative	Negative	Negative
9. Cyp2E1	Negative	Positive	Positive (centrilobular)	Negative/Positive
10. Cyp4A1	Negative	Positive	Positive (panlobular)	Positive

Negative/Positive indicate that 80% of the hepatocytes were negative and NA is not applicable.

Since DCA and TCA are formed as a mixture when TCE is metabolized, we are determining the tumor promoting activity of mixtures of the two chloroacetic acids. Female B6C3F1 mice were initiated on day 15 with 25 mg/kg of methylnitrosourea. At 7 weeks of age, they started to receive until sacrificed at 360 days of age, either DCA (1.0, 2.0 or 3.2gm/L), TCA (0.5, 1.0 and 4.0 gm/L) or mixtures of the two chloroacetic acids containing 1.0 gm/L TCA or 2.0 gm/L DCA with one of the three concentrations of the other chloroacetic acid. Promotion by DCA was demonstrated of foci of altered hepatocytes and adenomas and by TCA of adenomas and carcinomas. The mixtures also promoted foci of altered hepatocytes and adenomas. However, the presence of DCA reduced the adenoma and carcinoma response promoted by 1.0 and 4.0 gm/L TCA. The foci of altered hepatocytes and adenomas promoted by DCA were predominantly eosinophilic and positive for glutathione S-transferase- π (GST- π) and TGF α , but not TGF β . In contrast, the tumors promoted by TCA were basophilic, GST- π -negative and contained TGF α and β only in scattered immunopositive cells. The lesions promoted by the mixtures had characteristics resembling those promoted by DCA

At conclusion of this study, we will have determined:

- 1) the tumor-promoting activity of trichloroethylene;
- 2) the tumor-promoting activity of a mixture of two of trichloroethylene major metabolites, DCA and TCA, as a model for the carcinogenic activity resulting from the metabolism of trichloroethylene;
- 3) the differential sensitivity of precancerous lesions and non-involved hepatocytes to trichloroethylene and its metabolites with respect to enhancement of cell proliferation, proto-oncogene activation, and modulation of redox potential, metabolism and lipid peroxidation. The demonstration of a differential sensitivity of precancerous lesions compared to non-involved hepatocytes, would strongly support the proposed

- mechanism that trichloroethylene is carcinogenic in mouse liver by selectively expanding the size of the population of precancerous hepatocytes, and
- 4) the inhibition by trichloroethylene and its metabolites of apoptosis in precancerous lesions as demonstrated by a wave of apoptosis upon cessation of treatment with the test agents. The wave of apoptosis should occur in the precancerous lesions two days after the termination of treatment with trichloroethylene. In rats, the termination of treatment with other tumor promoters, resulted in the maximum increase in apoptosis at two days. Since these lesions compared to non-involved hepatocytes, also have an increased level of cell proliferation, the inhibition of apoptosis will further result in the selective expansion of the population of cells in these lesions, i.e. tumor promotion.

The effect of trichloroethylene upon non-involved hepatocytes and lesions is progressing. In the present study, we examined the expression of *c-fos*, *c-jun* and *c-myc* proto-oncogenes to determine whether they are deregulated in the process of TCE-induced tumors in mouse liver and kidney. B6C3F1 female mice were treated with 800 mg/kg TCE or corn oil vehicle via gavage, once daily at 5 days per week. After 1 or 300 days of exposure, the animals were sacrificed at 0, 45, 60, 90 and 120 min after the last dose. mRNAs of the proto-oncogenes were analyzed by northern blotting and the density calculated by a computerized image analysis system. After treatment with a single dose of TCE, the expression in the liver and kidney of *c-jun* increased from 0-90 min and then decreased by 120 min and of *c-myc* continued to increase throughout the 120 min evaluated. Expression of *c-fos* was not detected. After 300 days of 5 days/week treatment of TCE, the expression pattern of proto-oncogenes in the liver was similar to that after a single dose exposure of TCE except that *c-jun* expression peaked at 60 instead of 90 min. The results suggest that amplification and overexpression of *c-jun* and *c-myc* proto-oncogenes may play a role in TCE-induced carcinogenesis in the liver and kidney.

Dose-Response Relationship for Trichloroethylene: 56 Day study of trichloroethylene.

At Wright-Patterson, AFB the following study is being continued in collaboration with the Center for Environmental Medicine. Male B6C3F1 mice were administered five days a weeks and by gavage either 0, 0 + corn oil, 400, 800 or 1200 mg/kg bw trichloroethylene. Animals were sacrificed after 2, 3, 6, 10, 14, 21, 28, 35, 42, 49 and 56 days of treatment and the livers harvested and evaluated for cell proliferation (PCNA and mRNA histone-*in situ*), peroxisome proliferation, free radicals, lipid peroxidation, 8-hydroxyguanine levels, redox state, levels of ornithine decarboxylase and protein kinase C, and immunohistochemical quantitation of *c-fos*, *c-jun*, *c-myc*, TGF- α , and TGF- β 1. At the Medical College of Ohio, we will determine the level of apoptosis, glycogen content and synthesis, CYP2B1/2, CYP2E, CYP4A, glutathione S-transferase- π , and the expression of mRNA for *c-fos*, *c-jun* and *c-myc*. This study will provide the U.S. Air Force with the dose-response and temporal relationships of trichloroethylene with respect to three of the proposed mechanisms for the carcinogenic activity in mouse liver, i.e. enhanced cell proliferation, inhibition of apoptosis and formation of a mutagenic adduct in DNA (8-hydroxyguanine) resulting from oxidative damage. The results of this experiment will be compared to the initiation-promotion study in order to

- 1) to determine the relative sensitivity to modulation of molecular markers by trichloroethylene of acutely exposed hepatocytes, chronically exposed non-involved hepatocytes and precancerous hepatocytes in lesions;
- 2) to determine the ability of the molecular markers to predict the tumor promoting activity of trichloroethylene; and
- 3) to obtain an indication of the tumor promoting activity of trichloroethylene at dose levels lower than those suitable for use in the initiation-promotion study.

RESPIRATORY TOXICOLOGY DIVISION

The Respirable Toxicology Division has initiated this investigation to better understand the *in vivo* concentration dependency of bronchial epithelial cell toxicity to inhaled gases and particulates. The project's goals relate to both basic science investigations into the fundamental mechanisms of inhaled toxin dispersion throughout the respiratory tract, and subsequent intracellular markers of bronchial epithelial cell response, plus application of this information to specific toxins of concern to the U.S. Air Force and Navy. These studies are organized into two coupled efforts: 1. prediction of the concentration distribution of inhaled agents within the bronchial tree (human and small animal models) via computational simulation and experimental measurement of bronchial airflow convection patterns and the resultant aerosol propagation; plus 2. bronchial epithelial cell response (gene expression, quantitative cell morphology, protein excretion and cell surface markers of inflammation) to the local toxin concentration exposures predicted in part 1. Although these efforts are aimed toward general understanding of any inhaled gaseous and particulate toxin, we are specifically focused toward the potential of inhaled hydrazine and trichloroethylene gases (or as vapor aerosols) plus combustion product inhalation of fine particulate aerosols as potential bronchial carcinogens. In addition, we have initiated studies to evaluate the mechanisms of bronchial wall inflammation after smoke inhalation and the potential reasons for the high variation of systemic absorption of combustion products that are engendered through stimulation of regional pulmonary vasoreactivity.

Each of the molecular biological studies incorporate the local bronchial concentration dependence predicted by the aerosol dispersion analysis. The ultimate goal is to produce computational methods that can accurately predict *in vivo* inhaled toxin dispersion in the lungs of humans and animals. These methods are aimed toward describing the concentration-time exposures of regional respiratory epithelial cells plus predict the efficiency of systemic absorption for a wide variety of potentially toxic materials. These predictions then allow for more realistic cellular exposure experiments after biochemical and morphometric markers of toxin responses allow for sampling and analysis of the respiratory tract at specific locations characteristic of differing toxin exposures. It is hoped the predictions of inhalation dispersion, analysis of bronchial epithelial cellular markers, and methods of bronchial sampling after human or animal inhalation exposures can be applied to a wide variety of potential inhalation toxins in the future.

PROGRAM 1. Dispersion of Aerosol and Gaseous Toxins Throughout the Tracheobronchial Tree

As opposed to internal organ toxicity where a ubiquitous toxic exposure over relatively slow time constants is assumed, the respiratory tract can experience focused concentration of inhaled toxins for variable periods. The bronchial tree, being a highly efficient particle filter and soluble gas scrubber is designed to remove potentially harmful inhalants within the cascade of bronchial airways. This function may create accumulations of toxic substances at focal locations within the airways. In addition, the bronchial network can actively reorient the airstreams to divert irritants away from zones where focused irritants induce bronchoreactivity. The complexities of this system have to date only allowed only a general understanding of where materials accumulate in the respiratory tract and, as such, the specific concentration and duration of bronchial toxic exposure cannot be accurately determined. These constraints markedly limit understanding of inhalation injuries, for both inflammatory responses to inhaled injuries (as exemplified by bronchiolitis after smoke inhalation) and bronchial carcinogenesis.

Predicting the concentration distribution of toxins in the respiratory tract has been a subject of study for many years but with limited success. Recently, two important features of this phenomenon have been advanced affording a much better understanding of the bronchial particle filtering and gaseous scrubbing systems. Understanding of bronchial tree morphometry, especially for those geometrical features which influence airway aerodynamics, has been advanced. Such advancements have stimulated understanding of the unique air convective patterns within the airways characteristic to each zone of the bronchial tree. Current technology, using very large computer facilities, may allow application of the descriptions for each individual bronchial airstream into large arrays to simulate the entire bronchial network's function. Such simulation of the convective airstreams can then be used to predict gas dispersion plus aerosol propagation and deposition in the lung. Toxin deposition in the airways, when further coupled with mucus, lymphatic, and cough clearance from the airways, then predicts the toxin concentration-time exposures to the bronchial epithelial cells. We have initiated a comprehensive, multi-disciplinary effort to better understand the mechanisms of aerosol and gaseous distribution to the airway mucosa.

Three zones of the tracheobronchial tree are under investigation. Cast models of human and small animal upper and central airways have been produced and measures of airway flow mechanics obtained. These measures include air flow velocity fields and turbulence characteristics (kinetic energy and frequency spectra) throughout the upper and central airways. Using these empirical velocity patterns and turbulence characteristics, computational simulations of particle behavior and gaseous dispersions in the upper observed and central airways are in process.

Characterization of gas and particle dispersion in the bronchial network distal to the lobar bronchi presents a more challenging problem in that direct empirical measures of flow within the tiny airways is impossible. Fortunately, the bronchial tree has a complex but defined morphometry such that flow studies can be carried out in large scaled models of the small airways under conditions of fluid dynamic similitude (same ratio of forces acting on the flow).

To date, empirical flow studies done in airway models carefully mimicking the mid and small bronchi and bronchioles have been initiated. In addition, computer (numerical solutions) simulations of bronchial airway flow and aerosol conductance are under development using empirical measures from the upper and central airway casts (to predict aerosol conductance through the central airways) and impregnating this on the computer simulation of aerosol conductance and deposition throughout the lung. *In vivo* experiments to justify these predictions are also underway using tagged aerosols in small animal models. In addition, predictions of gaseous dispersion throughout the human tracheobronchial are being developed. The predictions will be similar to the predictions of aerosol conductance except that molecular diffusion of the convecting gas is added to the analysis. *In vivo* human experiments are underway to evaluate these predictions, measuring the dispersion of SF₆ and Helium between the pharynx and segmented bronchi plus from segmented bronchi to alveoli in several segments of normal human subjects.

This study is aimed at predicting the dispersion of gases and aerosols throughout the tracheobronchial tree, understanding the mechanism influencing this distribution, and evaluating the predictions through application to the *in vivo* human and small animal.

PROGRAM 2A. Gene Expression Related to the Metabolism of Toxins

Although two people may be exposed to the same toxin such as hydrazine or trichloroethylene, the risks each encounters due to the exposure may differ. In order to better assess these interindividual risks, we are evaluating the expression of genes that function in the metabolism of such toxins. Currently we are studying the expression of genes for xenobiotic metabolism enzymes in human bronchial epithelial cells. These cells are obtained from normal volunteers by bronchial brush biopsy. RNA from these cells is extracted using Tri-Reagent (Molecular Research Center, Inc.). Reverse transcription using oligo dT primers and murine reverse transcriptase is performed in order to obtain cDNA from the messenger RNAs present in the cells. Once cDNA is obtained, a recently developed technique known as multiplex competitive polymerase chain reaction (Apostolakos, *et al.*, 1993, Anal, Bio Chem. 213, 277-284) is applied to determine the levels of gene expression. This technique involves the use of a competitive template (CT) during PCR so that a ratio of CT to native gene product can be obtained. This ratio allows for better detection of expression because it remains constant even if the polymerase chain reaction reaches a plateau. In addition, a housekeeping gene is always amplified in multiplex with the gene of interest. This controls for the amount of cDNA loaded in each tube. Routine application of both of these controls makes this method both highly sensitive and reproducible.

Over the initial three months of this project, we have synthesized primers for amplification of the native gene products and for the preparation of the native competitive templates of nine xenobiotic metabolism enzyme genes. A list of these genes, primers, and the lengths of both the native product and CT is found in Table 1. Prior to completion of synthesis of all of the competitive templates for the xenobiotic enzyme genes, we initiated evaluation of bronchial brush cells and alveolar macrophages from volunteers using a semi-quantitative

method that included amplification of the native and CT of the housekeeping gene (-actin) and the native, but not the CT of the xenobiotic metabolism enzyme gene. This semi-quantitative method was used to evaluate expression of epoxide hydrolase (EH), phenol sulfotransferase (PST), N-acetyl transferase (NATA1), N-acetyl transferase 2 (NATA2), cytochrome p450 2F1 (CYP2F1), and cytochrome p450 2B7 (CYP2B7) in the bronchial epithelia cells from one volunteer. All of these genes were expressed. The relative amount of expression (in parentheses) using that of CYP2B7 as a standard equal to 1 was CYP2F1 (1.4 X), NAT1 (0.7 X), NAT2 (0.09 X), EH (0.97 X), and PST (0.97 X).

Most of the CT's have now been prepared. We will proceed with full multiplex competitive RT-PCR measurement of gene expression in additional volunteers and with other genes involved with the metabolism of toxins including those that may be found in products of combustion and in industrial air. We will sample human bronchial epithelial cells from a series of selected locations within the central bronchi and distal bronchioles. The sites will be selected to represent the spectrum of inhalation exposures predicted by our simultaneous aerosol and gaseous dispersion analysis. We expect to evaluate a total of 15 to 20 normal volunteers over the next year. In future years, we will seek to evaluate individuals with known exposure or risk for exposure to inhaled toxins.

GENE	NATIVE	CT	UPPER PRIMER SEQUENCE	POSITION
CYP1A1	355 bp	248 bp	5 ¹ CAT CCC CCA CAG CAC AAC AAG 3 ¹	1241
CYP2B7	352 bp	248 bp	5 ¹ GGA ACT TCG GAA ATC CAA GG 3 ¹	471
CYP2E1	338 bp	243 bp	5 ¹ TCG GCA TGG GGT TGG AGT TGT 3 ¹	1003
CYP2F1	362 bp	284 bp	5 ¹ GGG GAA GAG AAG CAT TGA GG 3 ¹	466
CYP3A4	373 bp	229 bp	5 ¹ TTG CTG GCT GAG GTG GTT GGG 3 ¹	24
EH	351 bp	258 bp	5 ¹ GGG TGA GAA CGT GGA GCC TG 3 ¹	31
NATA1	374 bp	275 bp	5 ¹ TAA GAG ATT CGC AGA GGC AAC CTG 3 ¹	113
NATA2	372 bp	271 bp	5 ¹ CTC CAA GGC CAC TGT TAG TTG TCA GA 3 ¹	17
PHENOL ST	349 bp	240 bp	5 ¹ CCG AAA TGC AAA GGA TGT GGT 3 ¹	440

GENE	NATIVE	CT	LOWER PRIMER SEQUENCE	POSITION
CYP1A1	355 bp	248 bp	5 ¹ ACA GCA GGC ATG CTT CAT GGT 3 ¹	1575
CYP2B7	352 bp	248 bp	5 ¹ CCA TGT GGA GCAGGT AGG TG 3 ¹	803
CYP2E1	338 bp	243 bp	5 ¹ AAT BCC CTC TTG CTA CTC GTC 3 ¹	1320
CYP2F1	362 bp	284 bp	5 ¹ GCC TGG TGG TCG TGG ACG CT 3 ¹	808
CYP3A4	373 bp	229 bp	5 ¹ CTC TGC TAT GCA TCC TCC TGA 3 ¹	376
EH	351 bp	258 bp	5 ¹ GGG TGA AAC GGA ACT TAT CG 3 ¹	362
NATA1	374 bp	275 bp	5 ¹ GTG TCT GAC CTC CCT TTC CAT TAT 3 ¹	463
NATA2	372 bp	271 bp	5 ¹ ATA TTT TAA TGG GAG TTG AAG GGA CA 3 ¹	363
PHENOL ST	349 bp	240 bp	5 ¹ GTG GGG ATG GTT GTG TAG TTA 3 ¹	768

GENE	NATIVE	CT	CT-PRIMER SEQUENCE	POSITION
CYP1A1	355 bp	248 bp	5 ¹ ACA GCA GGC ATG CTT CAT GGG TCT CAC CGA TAC ACT TCC G 3 ¹	1447
CYP2B7	352 bp	248 bp	5 ¹ CCA TGT GGA GCA GGT AGG TGGTGT GCC CCA GGA AAG TATT 3 ¹	679
CYP2E1	338 bp	243 bp	5 ¹ AAT GCC CTCTTG CTA CTC GTC CTG GTG AGG ATG GAG TTG GAC 3 ¹	1204
CYP2F1	362 bp	284 bp	5 ¹ GCC TGG TGG TCG TGG ACG CTC GGG AAT CTG GGG TCT AGGA 3 ¹	710
CYP3A4	373 bp	229 bp	5 ¹ CTC TGC TAT GCA TCC TTC TGA GCT GAT GGC TTG GTG GAA TAG 3 ¹	211
EH	351 bp	258 bp	5 ¹ GGG TGA AAC GGA ACT TAT CGG CCC CCA CCA CCC ATC TTC AA 3 ¹	248
NATA1	374 bp	275 bp	5 ¹ GTG TCT GAC CTC CCT TTC CAT TATCCC ACT TTC AAA GTA CAC TGCAAA T 3 ¹	339
NATA2	372 bp	271 bp	5 ¹ ATA TTT TAA TGG GAG TTG AAG GGA CA CAC TGA ATT CAA ACA AGG RGG TTA AA 3 ¹	236
PHENOL ST	349 bp	240 bp	5 ¹ GTG GGG ATG GTT GTG TAG TTA CCT TTT GGG GTT CTC CTT TAT 3 ¹	639

PROGRAM 2B. Bronchial Inflammatory Mediators of the Inhalation Injuries

We are developing molecular biological assessments of inflammatory indicators within the airway walls of small animals after exposure to hydrazine, trichloroethylene, and smoke inhalations from various combustions. The aim of this study is to enhance the understanding of the inflammatory pathways and develop cellular markers for the degree and persistence of inflammation as a function of the local airway dose-response and time-response of above described toxic chemicals. This effort will be extended to incorporate bronchoscopic airway samples obtained at bronchoscopy from humans exposed to low levels of hydrazine and trichloroethylene and smoke condensate in association with the gene expression studies described in 2A.

Our laboratory has extensive experience in the culture of ocular lens epithelial cells and regulation of gene expression of basement membrane molecules (collagenous and noncollagenous proteins) and their integrin receptors on exposure of epithelial cells to hormones, growth factors and cytokines (current NIH Eye Institute Study). These techniques have been extended to the bronchial epithelial cell analysis.

Epithelial damage in the airways is believed to be caused, in part, by the interaction of epithelial cells with leukocytes. Leukocytes must first adhere to and migrate through the endothelium, basement membranes, and epithelium before reaching the bronchial lumen. Adhesion molecules are important in this interaction and are upregulated or induced during an acute inflammatory reaction. The expression and modulation by hydrazine, trichloroethylene,

and smoke inhalation on adhesion molecules ICAM-1, LFA-3, and CD44 will be investigated in human and animal bronchial epithelial cells. Retinoids, glucocorticoids, transforming growth factors, and cytokines (TNF, IL-1) are also recognized to affect airway epithelial cells and will also be analyzed after exposure.

In a simultaneous experiment, we will also investigate the regulatory mechanisms of gene expression of basement membrane components (collagenous and noncollagenous proteins) in bronchial tissues or epithelial cells on exposure to above described toxins.

PROGRAM 2C. Pulmonary Microvascular Vasoreactivity in Relation to Systemic Absorption of Inhaled Toxins

Under this specific focus, we have initiated studies toward understanding the variation of systemic absorption of combustion products within smoke inhalation. We will investigate alterations of microvascular vasoreactivity as directed by the pulmonary macro- and microvascular endothelial cells. We will use a technique developed in our laboratory of assessing the output of vasoactive substances of endothelial cells in culture under a standard stimulus of graded hypoxia (16%, 8% O_2) with graded fluid shear stresses (high, medium, low flow states at the level of the precapillary arteriole).

Endothelial cells isolated from normal human pulmonary artery large vessel and microvascular sites are currently being propagated in culture. Measurements of the directed vasoreactivity of these cells will be examined via quantification of the release of endothelin -1 and 3 after hypoxia and shear. Quantitative multiplex competitive reverse transcriptase polymerase chain reaction (PCR) will be utilized to measure gene expression for endothelin which directly correlates to the levels of cytokine production. Once this model is established, several levels of endothelin release will be compared in the cells before and after exposure to LD₅₀ doses of hydrazine, trichloroethylene, and smoke representative of that inhaled from a class 1 fire.

Upon conclusion of this phase of testing, an animal model will be developed which will produce the above mentioned physiological conditions. The animals will be exposed to equivalent amounts of hydrazine, trichloroethylene, and smoke. Endothelial cells will be recovered from the pulmonary tract of the animal through the use of ULEX Europeans 1 coated Dynabeads™ in the methods described by Jackson *et. al.* PCR can again be used to determine from recovered endothelium the expression of endothelin -1 and 3.

PUBLICATIONS

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Four other publications in collaboration with Wright-Patterson Air Force base are in various stages of writing and will acknowledge this grant for its support. Reprints will be sent to AFOSR when received by us.