

Benzo[a]pyrene-Induced Immune Alteration in

the Japanese Medaka *Oryzias latipes*:

Probing the Underlying Cellular Mechanisms

by

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DEDICATION

To my family, Heather, Robert, Mom, and Dad.

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ABSTRACT

Increasing public concerns regarding the use of mammals in toxicological studies have led researchers to seek "alternative models" to investigate the biological effects of xenobiotics. Due to its small size and adaptability to a laboratory environment, the Japanese medaka (*Oryzias latipes*) lends itself well to such studies. Although medaka are used routinely for carcinogenicity testing, little emphasis has been placed upon this species for assessing the immunotoxic effects of chemicals. Given the known effects of benzo(a)pyrene (BaP) on the immune system of mammals, studies were conducted using medaka to examine the effects and cellular mechanisms by which exposure to BaP might alter the immune status of this laboratory fish model. Results demonstrated that IP treatment with BaP suppressed medaka lymphocyte proliferation, T-lymphocyte dependent antibody-forming cell (AFC) numbers, and phagocyte intracellular superoxide production. Thus, exposure of medaka to BaP resulted in suppression of both innate and adaptive immune parameters. Additional experiments demonstrated induction of CYP1A expression and activity within kidney tubules and mononuclear immune cells, indicating that medaka immune organs/cells are capable of metabolizing BaP into immunotoxic metabolites *in situ*.

Suppression of humoral immunity was not seen in medaka following exposure to the BaP congener, benzo(e)pyrene (BeP); BeP has low affinity for the aryl hydrocarbon receptor (AhR) and does not induce CYP1A in this system. Furthermore, co-exposure of medaka to BaP and compounds that inhibit CYP1A activity (i.e., α -naphthoflavone [ANF] or dehydroepiandrosterone [DHEA]) ameliorated the

immunotoxicity produced by exposure to BaP alone. This suggests that BaP metabolism into reactive metabolites may be required for suppression of immune function. *In vitro* exposure of medaka immune cells demonstrated the ability of BaP, BaP-7,8-dihydrodiol (BD), and BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) to suppress AFC numbers. Similar to that observed following *in vivo* BaP exposure, CYP1A inhibitors (i.e., ANF or ellipticine [ELP]) reversed BaP- and BD-induced immunotoxicity. The immunotoxicity observed following exposure to BPDE was not altered by ANF or ELP; suggesting that BPDE does not require metabolism to produce immunosuppression. Given that inhibition of CYP1A activity alleviates BaP-induced immunosuppression (both *in vitro* and *in vivo*), metabolism of BaP into its metabolites appears necessary for immunotoxicity in medaka.

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LIST OF ABBREVIATIONS

3-MC	3-methylcholanthrene
AhR	aryl hydrocarbon receptor
AIDS	acquired immunodeficiency syndrome
AIP	aryl hydrocarbon receptor interacting protein
ANF	α -naphthoflavone
ANOVA	analysis of variance
APCs	antigen presenting cells
ARNT	aryl hydrocarbon receptor nuclear translocator
BaP	benzo[a]pyrene
BCR	B-lymphocyte receptor
BeP	benzo[e]pyrene
BD	benzo[a]pyrene- <i>trans</i> -7,8-dihydrodiol (+/-)
BNF	β -naphthoflavone
BPDE	benzo[a]pyrene-r-7,t-8-dihydrodiol-9,10-epoxide (+/-) (anti)
BPQ	benzo[a]pyrene quinone
BSA	bovine serum albumin
BW	body weight
CFU	colony-forming unit
Con A	concanavalin A
CTL	cytotoxic T-lymphocyte

CYP1A(1)	cytochrome P450 1A(1)
DHEA	dehydroepiandrosterone
DHR	delayed-type hypersensitivity response
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	dimethyl sulfoxide
DNP	dinitrophenol
DPBS	Dulbecco's phosphate buffered saline
DRE	dioxin response element
ELISA	enzyme-linked immunosorbant assay
ELP	ellipticine
EROD	7-ethoxyresorufin <i>O</i> -deethylase
FBS	fetal bovine serum
FPS ⁺	fish physiological saline with glucose
FF	fixed-fluorescence
H&E	hematoxylin and eosin
HAHs	halogenated aromatic hydrocarbons
HBSS	Hank's balanced salt solution
HSP90	heat shock protein 90
ICC	immunocytochemistry
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin

IP	intraperitoneal
IT	intratracheal
KD	kilodalton
KLH	keyhole limpet hemocyanin
LALN	lung-associated lymph node
LD	lethal dose
LPS	lipopolysaccharide
MA	macrophage aggregate
MAF	macrophage activating factor
MDBA	7-methyldibenzanthracene
MHC	major histocompatibility complex
MLR	mixed-lymphocyte reaction
MNF	3'-methoxy-4'-nitroflavone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBT	nitroblue tetrazolium
NCC	non-specific cytotoxic cell
NK	natural killer
NTP	National Toxicology Program
SAR	structure-activity relationship
SCID	severe combined immunodeficiency
SOD	superoxide dismutase
PAHs	polycyclic aromatic hydrocarbons

PBS	phosphate buffered saline
PCBs	polychlorinated biphenyls
PCDDs	polychlorinated dibenzodioxins
PCDFs	polychlorinated dibenzofurans
PFC	plaque-forming cell
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
PMN	polymorphonuclear cell
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
O.D.	optical density
RAG	recombinase activating gene
RAST	radioallergosorbant test
RT	room temperature
s.c.	sub-cutaneous
SDS-PAGE	sodium dodecyl sulfate –polyacrylamide gel electrophoresis
SRBCs	sheep red blood cells
TBS	Tris-buffered saline
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TCE	trichloroethylene
TCR	T-lymphocyte receptor
TGF	transforming growth factor

TNF	tumor necrosis factor
TNP	trinitrophenol
TSA	tryptic soy agar
XRE	xenobiotic response element

I. PURPOSE, OVERALL HYPOTHESIS, & SPECIFIC AIMS

The purpose of this study was to evaluate the Japanese medaka (*Oryzias latipes*) as an alternative animal model to rodents for investigating immunotoxicology. Knowledge gained from studies examining the effects of chemicals upon the immune response of fish could, ultimately, be extrapolated to both mammalian (including human) and feral fish (teleost) populations. In addition, information gained from this study could aid in revealing previously unknown phylogenetically-conserved mechanistic responses to xenobiotics between fish and mammals.

The central hypothesis of this study was that benzo[a]pyrene (BaP) alters immune responses of medaka, and that BaP-induced alterations occur through cellular mechanisms similar to those thought to occur in mammalian species. BaP is a well-known mammalian immunosuppressant whose mechanisms of action have been well-researched. The hallmark of BaP-induced immunosuppression in mammals is suppression of T-lymphocyte-dependent antibody production (reviewed by White et al., 1994). Suppression of this and other immune endpoints appear to require cytochrome P450 1A1 (CYP1A1)-mediated metabolism of BaP into reactive immunotoxic metabolites. However, although teleost fish have been shown to possess an analogous system of BaP biotransformation (Stegeman and Hahn, 1994), little is known about the possible immunotoxic effects of BaP exposure in teleost fish species. It is hypothesized that BaP immunotoxicity is induced by comparable mechanisms in fish and mammals because of similarities in immune system components and BaP biotransformation pathways of both taxa.

Three Specific Aims were addressed to test the aforementioned hypothesis:

(1) To determine the effects of a single intraperitoneal (IP) injection of BaP on medaka immune functions, with particular emphasis on humoral immunity. Fish were injected once with graded, acutely non-toxic doses of BaP and analyzed by the performance of a battery of immune functional assays 48 h later. Kidney phagocyte respiratory burst activity was assessed to monitor effects upon innate immunity. Changes in T-lymphocyte proliferative responses were used as a marker of effects on cell-mediated immunity. T-lymphocyte-dependent antibody production and splenic B-lymphocyte proliferation were assessed to elucidate possible effects upon humoral immunity. Finally, changes in overall host immunocompetence were determined using a host resistance challenge assay.

(2) To assess whether CYP1A is induced in immune organs (kidney and spleen) following IP injection of BaP. Forty-eight hours-post BaP injection, CYP1A expression and enzyme activity were determined in both the kidney and spleen. CYP1A expression /activity in liver was assessed for use as a positive control because of its high metabolic activity. Expression of CYP1A protein was also determined for individual tissues and/or cell types within immune organs by immunohistochemical analysis. Finally, mononuclear kidney cells were isolated and CYP1A expression/activity determined for glass-adherent (i.e., macrophages) and non-adherent (i.e., lymphocytes) cell populations. Information gained by these studies determined whether BaP metabolism was occurring: (1) directly within specific

immune cell types; (2) by adjacent non-immune cells (i.e., kidney tubules); or, (3) at distant sites (i.e., liver) and metabolites either subsequently transported to immune organs or used to help generate immunomodulatory signals (i.e., indirect immunomodulation).

(3) To determine the role(s) that the aryl hydrocarbon receptor (AhR) pathway and CYP1A metabolism of BaP may play in BaP-induced

immunotoxicity. Effects of concurrent *in vivo* exposure to BaP and AhR antagonists /CYP1A inhibitors upon BaP-induced immunotoxicity in medaka were determined.

By inhibiting certain BaP biotransformation pathways, it was possible to assess the relative importance of either BaP metabolite production or AhR-mediated signal transduction in the observed immunotoxicity. For this Aim, fish were also exposed to a structurally-related chemical compound, but very weak agonist of the AhR benzo[e]pyrene (BeP). A lack of immunomodulation following exposure to BeP would suggest that non-metabolized BaP was not able to modulate the immune response by an AhR- and/or CYP1A-independent manner such as disrupting cellular membrane function. Furthermore, humoral immune function was also monitored following *in vitro* exposure of isolated immune cells to BaP and its metabolites.

Immunotoxicity following direct exposure to either the parent compound or certain BaP metabolites would rule out any indirect mechanisms of immunotoxicity (i.e., stress-related, sympathetic nervous system, endocrine). Simultaneous *in vitro* exposure to AhR antagonists /CYP1A inhibitors and BaP /BaP metabolites would likely reveal

the relative importance of AhR signaling and CYP1A-mediated metabolism of BaP directly within immune cells in BaP-induced immunotoxicity.

II. INTRODUCTION

A. The Immune System

1. Defending "Self" from "Infectious Non-self"

It is evident that the appearance of multicellular organisms (metazoa) was dependent upon the recognition of "non-self" entities and preventing them from infiltrating newly-established colonies. In addition, a system that efficiently removed abnormal cells from the organism would be essential. Immune responses are directly involved in both resistance against invading pathogens (i.e., infectious non-self) and maintenance of an internal homeostasis. Evidence for such a system exists even in the most primitive animals (i.e., phylum Porifera). Sponges possess the ability to create barriers around invading cells and produce cytotoxic factors; this response is akin to the process of allograft rejection in more complex organisms (Du Pasquier, 1993). However, immune systems have evolved considerably since the occurrence of the common ancestor of both modern sponges and vertebrates. Just as animals have become more sophisticated during evolution (i.e., nervous systems, body cavities, appendages, etc.), the phylogeny of immunity displays a similar trend of increasing complexity.

One of the most ancient responses of the immune system involves the non-specific recognition and elimination of pathogens, termed innate immunity. Innate

immune mechanisms are present in most animal phyla and include: anatomical barriers, antibiotic peptide molecules, and phagocytosis. However, some animals possess a much more complex compartment in their immune systems that relies upon specific genes whose products are capable of recognizing an unlimited array of molecular structures. This "adaptive immunity" incorporates specialized cells (i.e., lymphocytes) that react to the presence of "non-self" molecules.

The phylogenetic development of adaptive immunity apparently coincides with appearance of the vertebrate jaw. Modern jawless fish (i.e., lampreys and hagfish) appear to lack lymphocytes and adaptive immunity, while the most ancient of extant jawed vertebrates, cartilaginous fish (i.e., sharks, rays, and skates), possess all of the components of adaptive immunity. The common ancestor of these two groups, presumably the precursor of fossilized jawed placoderms, existed approximately 500 million years ago (Litman, 1996). Thus, within the relatively short period of time between the development of the jaw (500 million years ago) and divergence of sharks (450 million years ago), this highly complex immune system arose (Bernstein et al., 1998). Although the exact evolutionary pressures leading to adaptive immunity remain unknown, one current theory states that it arose in the gut of primitive jawed fish due to a newly acquired predatory life-style leading to increased digestive tract injury and subsequent infections (Matsunga and Rahman, 1998). Whatever the phylogenetic explanation, adaptive immunity has become so important in humans that the removal of this immune arm greatly compromises survival.

2. Components of the Mammalian Immune System

The purpose of this section is to provide a brief overview of the mammalian immune system so as to provide a basis for comparison for these studies.

Components of the mammalian immune system are tightly regulated by both internal (i.e., cytokines) and external (i.e., neuroendocrine) signals. In addition, the immune system is located throughout the body within several lymphoid organs (i.e., thymus, bone marrow, spleen, lymph nodes); various immune cell types including lymphocytes, macrophages, neutrophils are involved. Although many components of the immune system rely upon inter-immune response interactions, mammalian immunity can be broadly divided into two major compartments: innate (non-specific) and adaptive (specific). Common to both compartments is the necessity for recognition and elimination of foreign structures while producing as little damage as possible to the host.

The first lines of defense in mammalian immunity involves anatomical, biochemical, and physiological barriers. Anatomical barriers include the skin epidermis and the mucus membranes of the respiratory, digestive, and urinary tracts. These barriers also provide biochemical /physical constituents that impede pathogen penetration such as mucus, lysozyme, low pH, and coughing reflexes. Besides the initial lines of defense, the host also has several cellular and soluble innate mechanisms to deal with pathogen invasion. One of the innate non-cellular mechanisms is the acute phase response which is activated by inflammation and involves the release, mainly by hepatocytes, of various proteins (i.e., C-reactive

protein, serum amyloid P, serum amyloid A). These proteins aid in the destruction of pathogens and possess reactivity towards evolutionarily-conserved molecular motifs expressed by many microorganisms. In addition, anti-viral interferons are released by many cell types following infection. Soluble innate components that are expressed at high levels prior to infection include serum lysozymes and complement that can be subsequently activated by adaptive immunity.

Cellular components of innate immunity include anti-tumor and phagocytic cells. Anti-tumor cells (termed “natural killer” or NK cells) possess the capability to recognize and destroy cells expressing viral or tumor antigens. Although they are thought to be closely related to T-lymphocytes, NK cells function in the destruction of neoplastic cells in a relatively non-specific manner (i.e., no immunological memory required). NK activity results in the release of cytotoxic (apoptosis-inducing) factors directed against the target cell.

Phagocytic cells can be divided into granulocytes and cells of the monocyte / macrophage lineage. Granulocytes include specialized polymorphonuclear (PMN) neutrophils that are usually the first immune cells to arrive at the site of inflammation by exiting blood vessels and infiltrating infected tissues. PMN activation leads to the production and release of bactericidal factors from their numerous cytoplasmic granules, often resulting in tissue damage. In addition, PMN are effective phagocytes involved in the uptake and intracellular destruction of microorganisms.

While macrophages, terminally-differentiated tissue monocytes, are often described as professional phagocytes, their function in the immune system goes well

beyond phagocytosis. Macrophages permanently reside in many tissues including the liver (Kupffer cells), brain (microglial cells), and lung (pulmonary macrophages). Both resident macrophages and monocytes possess diverse arrays of surface receptors involved in the identification of pathogens (i.e., lectin receptors) and regulation of the immune response (i.e., cytokine receptors). In addition, macrophages express molecules on their surface specifically required for the generation of an adaptive immune response. These molecules, termed major histocompatibility complex (MHC) class II, are expressed in-conjunction with small peptides derived from phagocytosed foreign material (i.e., antigen). The recognition of these molecules by lymphocyte receptors specific to the presented antigens results in proliferation and differentiation of antigen-specific lymphocytes. This function, not specific for macrophages alone, is termed "antigen presentation."

Although components of innate immunity are capable of recognizing foreign structures expressed by invading pathogens, the adaptive branch of immunity reacts in a more specific manner. Adaptive immunity has the capability of memory (i.e., remembering an antigen previously encountered). Once a pathogen is recognized for a second time, an adaptive immune response is activated that is both more intense and rapid than the initial one. Adaptive immunity, relying upon immune genes expressed only in specialized cell types (i.e., lymphocytes), can be divided into two major branches: cell-mediated and humoral immunity.

Lymphocytes rely upon receptors that bind antigen (recognizable molecules on foreign organisms or their products). These receptors (i.e., antibodies, T-cell receptors

[TCRs], and MHCs) have a common molecular structure (i.e., the immunoglobulin-fold) and are capable of binding to an enormously diverse array of molecules.

Antibody and TCR genes rely upon somatic germline recombination and other genetic phenomena to create this diversity. Mammals use these receptors to ultimately define “self” from “non-self” in a manner much more specific than that done in innate immunity. Lymphocytes possessing receptors reactive to “self” antigens are removed during lymphopoiesis. This lymphocyte “education” occurs within the primary lymphoid tissues (i.e., bone marrow and thymus gland).

Not only is an effector function carried out initially upon the recognition of foreign antigen, but lymphocytes possessing the antigen-binding receptor then proliferate; some become the memory cells that are present upon subsequent exposure to antigen and, thus, greatly decrease the response time. The exact antigen-induced effector mechanism depends upon the type of lymphocyte activated.

The two major forms of lymphocytes are B- and T-cells. Upon activation, B-lymphocytes (B = bursa) produce soluble antibodies which are involved in the coating (opsonization) and eventual destruction of foreign microbes (i.e., humoral immunity). Antibodies are of the immunoglobulin (Ig) superfamily and exist as two major forms; membrane-bound (B-cell antigen-detecting receptor; BCR) and soluble forms secreted upon B-cell activation. The antigen-binding specificity of the BCR is created by germline rearrangements and is nearly identical to soluble antibodies produced by that same cell. Secreted antibodies can activate complement molecules in the serum via the classical complement pathway. The activation of complement leads to further

opsonization of foreign objects to allow for recognition by immune cells. Complement also has the ability to lyse target cells via a membrane attack complex and attract cells to the site of inflammation by chemotaxis.

T-lymphocytes (T = thymus) exist as two major types; those that help activate B-cells to produce antibody (i.e., T helper cells; T_h) and those that destroy infected / infectious cells directly (i.e., T cytotoxic cells; T_c). There are also T-lymphocytes that suppress the generalized immune response (T_s) and are involved in delayed-type hypersensitivity reactions (T_{DTH}). All mature T-cells possess antigen-specific receptors distinct from BCR, termed T-cell receptors (TCRs). TCRs also undergo germline recombination (similar to BCR) and are directly involved in the selection of T-cells during "thymic education." TCRs recognize antigens when expressed on the surface of cells in conjunction with MHC molecules.

MHCs are also members of the Ig superfamily. The binding capabilities of MHC are less specific than those of BCR and TCR. Peptides present within cells are combined with MHC molecules and then expressed on the cell surface. Endogenously- and exogenously-derived proteins are presented in combination with MHC class I and II antigens, respectively. BCR or TCR co-receptors then recognize these complexes. If a B-cell expresses a receptor that binds to the presented peptide, the cell is then activated to produce soluble antibodies specific only for this particular antigen (i.e., humoral immunity). If a T-lymphocyte recognizes the complex, the final result will depend upon the T-lymphocyte subpopulation involved. T_h -lymphocytes are only reactive to a certain subset of cells expressing MHC type II molecules (termed antigen

presenting cells; APCs). However, T_c -lymphocytes are reactive to MHC type I molecules which are expressed by many cell types. Activated T_h lymphocytes release various cytokines to stimulate the immune system and also aid in the activation of B-cells. Activated T_c -lymphocytes destroy cells expressing a recognizable foreign antigen (i.e., cell-mediated immunity).

Despite the obvious differences between innate and adaptive immunity, important interactions occur between the two disparate compartments. Products of the adaptive immune response greatly enhance innate immune responses such as phagocytosis (i.e., opsonization). In addition, recent studies in mammals have revealed that activation of innate immunity in response to infection is involved in directing the adaptive immune response (Janeway, 2001). Add to this, indirect mechanisms of immunomodulation such as those involving the endocrine system and hypothalamic-pituitary axis, and one can just begin to envision the complexity of the immune response.

3. *Mammalian Immunotoxicology*

Immunotoxicology is defined as the study of injury to the immune system by environmental chemicals (natural or anthropogenic) or pharmaceuticals (NRC, 1992). Altered immune function can result in either enhanced (i.e., hypersensitivity and autoimmunity) or suppressed (i.e., immunodeficiency) immunity. Although the adverse effects of overactivation of the immune system are often obvious (i.e., allergy

and autoimmune disease), immunosuppression can be more subtle resulting in an increased prevalence of infectious disease and/or neoplasia.

a. Immune-Mediated Disease

The occurrence of hypersensitivity in humans following environmental or occupational exposure to xenobiotics is well-documented (reviewed by Burns-Naas et al., 2001). Evidence exists for immediate (type I), antibody-dependent cell cytotoxic (type II), immune-complex-mediated (type III), and delayed-type (type IV) hypersensitivities in humans following exposure to such compounds as penicillin, heavy metals (i.e., nickel, platinum, beryllium, and chromium), cosmetics, and latex. The prevalence of xenobiotic-induced autoimmunity in humans is highly-dependent upon both environmental and genetic factors. Various chemicals have been associated with autoimmunity such as vinyl chloride, mercury, and silica (Burns-Naas et al., 2001), although their exact roles in the observed autoimmune disorders are not fully understood.

b. Immunosuppression and Host Resistance to Infectious Disease and Neoplasia

The potential of chemicals to suppress the immune response and subsequently increase susceptibility to pathogens and/or neoplasia has been studied for over three decades (Koller, 2001). However, immunotoxicology did not gain credibility until, in the early 1980s, investigations revealed that xenobiotics (i.e., toxaphene, pentachlorophenol, lead, and polychlorinated biphenyls) could induce

immunosuppression in rodents at doses below those that altered more classic toxicological endpoints (Allen et al., 1982; Kerkvliet et al., 1982; Koller et al., 1982). From these early immunotoxicological studies one observation became apparent - linking data obtained in rodent models to human populations has been extremely difficult. Such extrapolation must assess differences between humans and rodents in chemical dose and toxicokinetics, as well as differences in susceptibility and exposure history within human populations. Determination of the magnitude of immunosuppression (in both rodent models and/or humans) that is required for increased susceptibility to disease or cancer is essential for accurate human risk assessment.

The best evidence demonstrating the link between immunosuppression and increased susceptibility to infectious disease and/or neoplasia in humans comes from studies of individuals with immunodeficiency disease (inherited or acquired) or those patients receiving immunosuppressive therapeutics. An increased incidence of bacterial, viral, fungal, and/or protozoal infections have been documented in individuals with congenital defects in innate- (i.e., complement deficiency), humoral- (i.e., X-linked agammaglobulinemia), or cell-mediated immunity (i.e., DiGeorge syndrome), or combinations of various immune compartments (i.e., severe combined immunodeficiency disease [SCID])(reviewed by Kuby, 1994). Furthermore, individuals with acquired immunodeficiency syndrome (AIDS) express humoral and cell-mediated immunosuppression and increased incidence of *Pneumocystis carinii* infection, Kaposi's sarcoma, and other rare disorders. Finally, treatments involved in

organ transplantation or cancer chemotherapy, such as X-irradiation or immunosuppressive drugs, have been associated with increased risk of infection and/or cancers such as non-Hodgkins lymphoma and Kaposi's sarcoma. Similar associations between immunosuppression and disease susceptibility have been made in rodent models exhibiting inherited immunodeficiencies (i.e., nude, SCID, beige, and various knockout mice) or physically/chemically-disrupted immune compartments (i.e., irradiation of bone marrow or thymus).

There is also mounting evidence regarding the potential of a variety of environmental chemicals (natural and anthropogenic) to induce immunosuppression. However, clear-cut examples of chemical-induced immunosuppression in humans are rare (Burns-Naas et al., 2001). Although some chemicals have been found to alter immune parameters in exposed humans (i.e., aldicarb, TCDD, benzene, oxidant gases, methyl isocyanate, ultraviolet radiation, ethanol, and cocaine), correlations with clinical disease are not always apparent (NRC, 1992; Vos and van Loveren, 1998). In general, examples of xenobiotic-induced immunosuppression in association with increased disease incidence in humans are limited to either occupational or accidental exposures (Vos and van Loveren, 1998). For example, decreased serum antibody levels; peripheral T-lymphocytes; and, delayed-type hypersensitivity observed in Taiwanese people (Yu-Cheng disease) exposed to rice oil contaminated with polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) were associated with an increased incidence of skin lesions, liver damage, and sino-pulmonary infections (Chang et al., 1981, 1982; Wu et al., 1984; Lee and Chang,

1985). Associations between clinical disease and immune dysfunction following occupational exposures to lead (Ewers et al., 1982; Jaremin, 1983; Cohen et al., 1989) and silica (Uber and McReynolds, 1982) have also been established. However, in many instances the correlation between immunosuppression and clinical disease in human populations is subtle and difficult to prove, (i.e., increased incidence of leukemia and infections in children associated with trichloroethylene (TCE)-contaminated drinking water of East Woburn, MA; Lagakos et al., 1986; Byers et al., 1988). To complicate matters even further, isolated incidences of human exposure to heavy metals or TCDD (at levels above those known to result in rodent immunotoxicity) fail to alter immune parameters (NRC, 1992). However, many of the clinical studies monitoring immune function following xenobiotic exposure suffer from small sample size, lack of reproducibility, and inaccurate determinations of exposure levels (Vos and van Loveren, 1998).

c. Assessment of Immunotoxicity in Humans and Rodent Models

The classic animal models for investigating immunosuppression are rodents, particularly mice. The mouse model is well-characterized immunologically, with various agents such as cytokines, cell lines, and monoclonal antibodies commercially available. In addition, mice are relatively inexpensive (compared to other rodent species) and easily maintained in the laboratory. However, the rat model has been most commonly used in routine toxicological research. Besides the rat and mouse,

other mammals such as guinea pigs, rabbits, and non-human primates have been utilized for immunotoxicology studies.

Chemically-induced hypersensitivity can be broadly categorized into two major groups: (1) dermal (contact) hypersensitivity, and (2) respiratory hypersensitivity (Karol, 1998). Contact hypersensitivity is a cell-mediated, delayed-type hypersensitivity response (type IV); tests for contact allergens include: the Buehler test (Buehler, 1995); guinea pig maximization test (Magnusson and Kligman, 1969); local lymph node assay (Kimber, 1995); and, mouse ear-swelling tests (Gad, 1995). Humans can be tested for contact hypersensitivity by the use of a patch test (Burns-Naas et al., 2001). Respiratory hypersensitivity is thought to be an immediate (type I) hypersensitivity reaction involving the production of IgE antibodies. Guinea pig, rat, and mouse models have been utilized to assess chemically-induced respiratory hypersensitivity (Burns-Naas et al., 2001). Furthermore, humans can be tested for IgE-mediated reactions by serological tests (i.e., ELISA and radioallergosorbent tests [RASTs]) or skin patch tests that measure a classic "wheal and flare" reaction (Burns-Naas et al., 2001).

Although many chemicals are suspected of having a role in the induction of autoimmunity either directly (causative agent) or indirectly (as an adjuvant), very few animal models exist to test this hypothesis. Three animal models most commonly used for these purposes are the murine popliteal lymph node assay, human lymphocyte transformation, and graft-vs-host models (Burns-Naas et al., 2001). In addition, anti-"self" antigen antibodies can be detected both in the serum and immunohistologically.

In order to better extrapolate murine immunotoxicological data to human risk assessment, Luster et al. (1988) proposed a battery of immune assays useful for identifying potential immunosuppressive chemicals. Standardization of these assays was achieved by a coordinated effort, between laboratories of the National Toxicology Program (NTP), the National Institute of Environmental Health Sciences (NIEHS), and the Chemical Industry Institute of Toxicology (CIIT) that generated a database of immunotoxicological assessments for over 50 different compounds (Luster et al., 1992).

The NTP's approach for investigating rodent immunotoxicity includes two tiers and is outlined in Table 1. Tier I was designed to determine the immunotoxic potential of compounds at doses that are not acutely toxic. Tier II investigates more subtle effects on humoral, cell-mediated, and innate immune functions along with host resistance against pathogens and tumor challenge. In the battery of assays, two tests possessed high individual predictive values for overall immunotoxicity: enumeration of lymphocyte subpopulations (83% correct predictions) and the plaque-forming cell (PFC) assay (78% correct). Pair-wise combinations of either one of the aforementioned assays with any other assay in the battery greatly increased the predictive value, and some 3-test combinations yielded 100% concordance (Luster et al., 1992). The correlation between alterations observed in immune functional assays and host resistance was also evaluated (Luster et al., 1994). These studies revealed that chemical exposures which failed to alter immune function also had no effect upon host resistance. Tests for altered lymphocyte surface marker expression, delayed-type

Table 1.

Two-tiered approach for identifying immunotoxic compounds in rodent models^a

Parameter	Procedures
<i>Tier I</i>	
Immunopathology	Blood differentials Immune weights Immune organ cellularity Histopathology
Innate immunity	Natural killer (NK) cell activity
Cell-mediated immunity	T-lymphocyte blastogenesis Mixed lymphocyte response (MLR)
Humoral-mediated immunity	Enumeration of primary plaque-forming cells (PFCs) LPS-stimulated B-lymphocyte proliferation
<i>Tier II</i>	
Immunopathology	Quantitation of splenic B- and T-lymphocyte numbers
Innate immunity	Macrophage functional assays
Cell-mediated immunity	Cytotoxic T-lymphocyte (CTL) response Delayed-type hypersensitivity (DTH) response
Humoral-mediated immunity	Enumeration of secondary plaque-forming cells (PFCs)
Host Resistance Challenge	Tumor resistance (i.e., PYB6 sarcoma) Bacterial resistance (i.e., <i>Listeria monocytogenes</i>) Viral resistance (i.e., influenza) Parasite resistance (i.e., <i>Plasmodium yoelii</i>)

^aTable adapted from Luster et al. (1988).

hypersensitivity response (DHR), and PFC assays produced high associations with altered host resistance. Pair-wise combinations that included PFC, surface marker, or DHR assays also resulted in a very high concordance value with host resistance. The remainder of this section briefly describes each of the immune assays used to assess immunosuppression and host resistance (reviewed by Burleson et al., 1995).

In general, assessment of innate immune function involves analysis of NK cells, macrophages, and PMN. However, assays are occasionally performed that measure soluble innate molecules such as interferon (Burleson and Burleson, 1995), acute phase proteins (Kaminski, 1995), and complement (Lin and White, 1995). Assays that assess rodent NK cell activity measure their ability recognize and lyse YAC-1 tumor target cells in a ^{51}Cr release assay (Djeu, 1995); human NK cell activity can be assessed in a similar procedure using K562 tumor cells (Burns-Naas et al., 2001). Macrophage function can be evaluated by examining such activities as chemotaxis, adherence, phagocytosis, respiratory burst, anti-tumor activity, and intracellular bacterial killing (Lewis, 1995; Neldon et al., 1995; Qureshi and Dietert, 1995; Rodgers, 1995). It is also important to determine xenobiotic-induced effects upon macrophage activation following either stimulation *in vitro* (i.e., phorbol ester, zymogen, macrophage activating factor [MAF]) or elicitation *in vivo* (i.e., *Corynebacterium parvum* or thioglycolate) (Luster et al., 1988). In addition, the antigen presenting capability of macrophages can be determined using radiolabeled antigens. PMN activity can also be evaluated by examining chemotaxis, phagocytosis,

and respiratory burst activity; functional assays can be performed using human PMN and monocytes/macrophages from blood or lung lavage.

The humoral immune response of rodents is usually assessed by determination of B-lymphocyte blastogenesis and T-cell-dependent antibody production. The ability of B-cells to proliferate following stimulation is essential for the generation of a robust antibody response. Immunotoxicological studies measure B-cell proliferation following *in vitro* stimulation with either the polyclonal B-cell mitogen lipopolysaccharide (LPS) or a combination of interleukin (IL)-4 and anti-surface Ig (Burns-Naas et al., 2001). Quantitation of proliferation is performed by ^3H -thymidine incorporation into DNA or using non-radioactive assays (i.e., MTT); protocols identical to those used to assess B-cell blastogenesis in a murine model can also be used with human peripheral blood leukocytes.

An assessment of the primary (IgM) antibody response in rodents is generally performed by an antibody, plaque-forming cell (PFC) assay (reviewed by Holsapple, 1995). In this assay, animals are immunized *in vivo* with the T-dependent antigen sheep red blood cell (SRBC). A primary humoral response is generated at approximately 4 –5 days post-immunization. At this point animals are sacrificed and immune cells isolated from the spleen prior to their combination with the antigen (i.e., SRBC) in the presence of complement in an agarose vehicle. The mixture is allowed to harden in petri dishes for 3 h. This results in visible plaques created by complement-mediated lysis of SRBCs any surrounding antibody-secreting B-cells.

Plaques are then enumerated to determine the relative quantity of plaque-forming cells per spleen (or per the number of splenocytes per mixture).

Two major variations in the exposure /immunization protocol of the primary PFC exist. Animals can be exposed to a chemical *in vivo* followed by *in vitro* immunization of splenocytes (Holsapple, 1995). In addition, an *in vitro* PFC (i.e., Mishell-Dutton) assay can be performed by isolating splenocytes from naive animals prior to *in vitro* chemical exposure and immunization (Mishell and Dutton, 1967). Analysis of the secondary immune response (IgG) can also be performed with slight modifications of the IgM PFC protocol (Holsapple, 1995). Soluble T-dependent antigens (i.e., keyhole limpet hemocyanin [KLH]) or haptenated-antigens (KLH-dinitrophenol) can be utilized in PFC assays to further characterize the humoral immune response. Antigens that do not require T-cell help (T-independent antigens) such as DNP-Ficoll (i.e., only requires macrophages and B-cells) or LPS (i.e., only requires B-cells) have also been successfully incorporated into the PFC protocol. Finally, various separation / reconstitution techniques have been employed using PFC assays to determine those specific cell type(s) (i.e., T-cells, B-cells, or macrophages) affected by xenobiotic exposure (Holsapple, 1995). Alternative assays for assessing humoral immunity include ELISAs for detecting serum anti-antigen antibodies (Temple et al., 1995) and ELISPOT assays which can be used to enumerate antibody-forming cells in a microtitre plate assay (Kawabata, 1995).

Cell-mediated immunity can be assessed by T-cell blastogenesis, mixed lymphocyte reaction (MLR), cytotoxic T –lymphocyte (CTL), and DHR assays. The

proliferation of T-cells following exposure to mitogens such as concanavalin A (Con A) and phytohemagglutinin (PHA) can be determined in a manner similar to that described for B-lymphocyte proliferation (reviewed by Smialowicz, 1995). The lymphoproliferative response between allogeneic spleen cells can be assessed by a one-way MLR (reviewed by Smialowicz, 1995); this response is similar to cell-mediated graft rejection or graft versus host reactions (Luster et al., 1988). Briefly, two populations of splenocytes (one treated with mitomycin C to prevent proliferation) are mixed together and the proliferative response of the untreated (no mitomycin C) population is quantitated by ^3H -thymidine uptake. The CTL assay measures the ability of cytotoxic T-lymphocytes to recognize and destroy allogeneic cells (House and Thomas, 1995). Splenocytes are first sensitized with mitomycin C-treated P815 mastocytoma cells (allogeneic cell population). CTLs differentiate and proliferate during this period in response to target cells expressing foreign antigen (i.e., MHC restriction). After sensitization, CTLs are isolated and incubated with radiolabeled (^{51}Cr) target cells; the release of radioactivity from cells lysed by antigen-specific CTLs is measured. Finally, the DHR assay measures a type IV hypersensitivity reaction involving CD4^+ memory T-cells (Burns-Naas et al., 2001). In this assay, rodents are sensitized by subcutaneous injection of KLH. Following the sensitization period, animals are injected intravenously with ^{125}I -5-iododeoxyuridine (IUdR). The radionucleotide is then incorporated into proliferating monocytes and after 1 d animals are injected in a single ear with KLH. The following day, ears are biopsied for the determination of monocyte infiltration into the KLH-injection site.

Host resistance assays are often performed in animal studies to assess altered susceptibility to pathogen infection or tumor growth following exposure to an immunotoxic compound. Caution should be used, however, when interpreting host resistance data since alterations in susceptibility may be due to effects other than immunosuppression (i.e., the direct effect of the chemical compound upon the pathogen). Most host resistance assays involve the exposure of animals to infectious pathogens or tumor cells which results in either 30% cumulative mortality (i.e., LD₃₀) or 30% of the animals showing tumor incidence /growth, respectively. Thus, both increased and reduced host resistance to challenge can be determined following xenobiotic exposure. Challenge agents commonly employed in mammalian immunotoxicological studies include bacteria (i.e., *Listeria monocytogenes*, *Streptococcus pneumonia*), protozoa (i.e., *Plasmodium yoelii*), nematodes (i.e., *Trichinella spiralis*), viruses (i.e., influenza virus and murine cytomegalovirus), and syngeneic tumor cells (i.e., B16 F10 melanoma and PYB6 sarcoma) (Luster et al., 1988; Bradley, 1995).

4. Fish Immunology

Most immunological studies employing fish focused on species of phylogenetic or economic importance. Thus, such groups as agnathans, elasmobranches, and chondrosteans have been studied in an attempt to elucidate evolution of the vertebrate immune system. Also, species such as salmonids (*Oncorhynchus spp.* and *Salmo spp.*), carp (*Cyprinus spp.*), and catfish (*Ictalurus spp.*)

have received interest mainly due to their use in aquacultural settings. Therefore, out of greater than 25,000 fish species, relatively few have been the subjects of immunological investigations. Several excellent reviews of the current status of fish immunology exist including those by Iwama and Nakanishi (1996), Bernstein et al. (1998), and Patoret et al. (1998).

a. Leukocytes & Lymphoid Organs / Tissues

Fish leukocytes possess many morphological and functional similarities with their mammalian counterparts (reviewed by Rowley et al., 1988). Fish equivalents to mammalian macrophages (Secombes and Fletcher, 1992; Secombes, 1996), granulocytes (Ainsworth, 1992; Secombes, 1996), non-specific cytotoxic cells (NCCs) [forerunners to mammalian NK cells; Evans and Jaso-Friedmann, 1992; Secombes, 1996], T-lymphocytes (Manning and Nakanishi, 1996; Partula, 1999; Scapigliati et al., 1999), and B-lymphocytes (Kaattari and Piganelli, 1996) have been identified.

While the location and organization differs somewhat from mammalian lymphoid tissue, teleost fish possess a variety of lymphoid tissues (reviewed by Zapata et al., 1996). The primary site of hematopoiesis /lymphopoiesis in fish is the anterior portion of the kidney (also called pronephros or head kidney). Generally, the kidney is located along the dorsal wall of the body cavity in most species. It not only contains hematopoietic tissue, but also houses medullary (chromaffin cells) and cortical (interrenal cells) adrenal homologues, stanniocalcin-secreting tissue (corpuscles of Stannius), and renal excretory machinery (Panel 1A). Studies have concluded that the

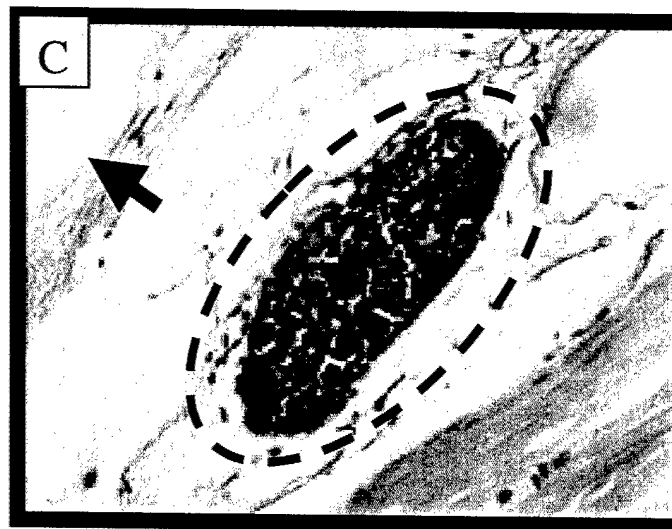
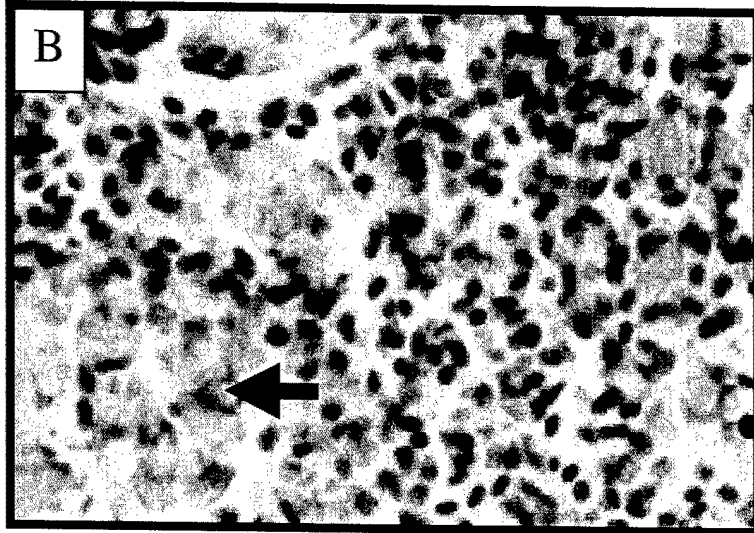
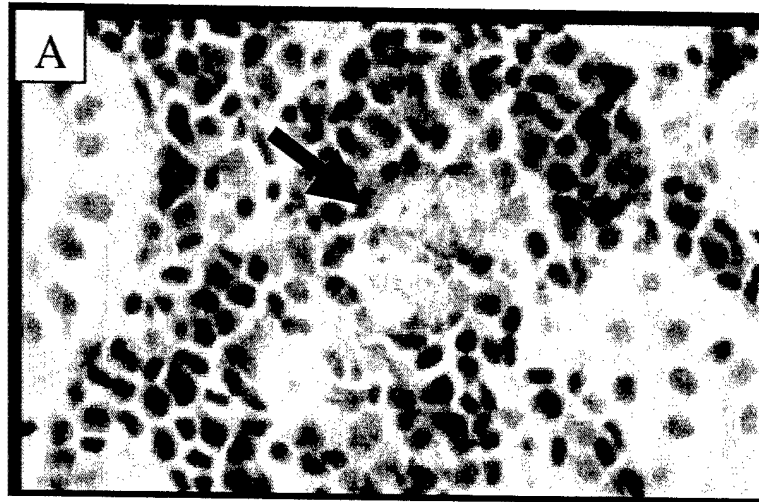
Panel 1. Light microscopy of medaka lymphoid tissues displaying normal histology.

(A). Hematopoietic tissue of the medaka kidney; arrow = macrophage aggregate. (B)

Histological appearance of the medaka spleen; arrow = macrophage aggregate. Note the lack of well-developed germinal centers. (C) Histological appearance of the

medaka thymus (within dashed circle); arrow = opercular epithelium. (original

magnifications: A – B, 1000X; C, 100X)



pronephros is the first organ to become populated with B-lymphocytes during ontogeny (Romano et al., 1997).

Teleost fish also possess a thymus gland located dorsolaterally within the opercular cavities (reviewed by Chilmonczyk, 1992). Histologically, the thymus is poorly-differentiated into cortical and medullary regions (Panel 1C; Zapata et al., 1996). As found in mammals, the fish thymus is the first organ to become populated with lymphocytes during development and appears to involute with age and exposure to certain environmental stimuli (Chilmonczyk, 1992; Fishelson, 1995; Alvarez et al., 1998).

While fish lack lymph nodes, the spleen is a secondary lymphoid organ in fish (Zapata et al., 1996). Although lacking the highly-developed germinal centers observed in mammalian spleens, the teleost spleen houses many immune cells found in both red and white pulps (Panel 1B; Press et al., 1994; Zapata, 1982; Quesada et al., 1990).

Teleosts possess highly diffuse and unorganized lymphoid accumulations consisting of lymphocytes, granulocytes, macrophages, and plasma cells, throughout the gut, gills, and skin (Hart et al., 1988; Kaattari and Piganelli, 1996; Zapata et al., 1996; Abelli et al., 1997). This mucosal lymphoid tissue appears to possess a distinct subpopulation of T-lymphocytes that are observed very early during development (Rombout et al., 1998). Numerous studies have described antigen uptake by the gut (Rombout et al., 1985), skin, and gills (Moore et al., 1998). In addition,

immunological responses such as “oral tolerance” have been described in the mucosal immune systems (Kaattari and Piganelli, 1996; Jones et al., 1999).

b. Innate Immunity

Skin plays an important role in non-specific immunity of fish. Unlike that observed in mammals, fish skin lacks a stratum corneum consisting of dead cells. Rather, the epidermis of fish is composed solely of non-keratinized living cells which makes wound healing in fish extremely rapid. In addition, teleost skin possesses a mucus covering found to contain natural antibodies, lysozyme, complement, and bacteriolysins (Zapata et al., 1996). Many soluble molecules involved in mammalian innate immunity have also been described in fish including complement (reviewed by Sakai, 1992; Yano, 1996), interferon (reviewed by Yano, 1996), lysozyme (reviewed by Yano, 1996), and acute phase proteins (Yano, 1996; Magor and Magor, 2001); many of which appear to enhance phagocytosis by serving as opsonins.

The morphology and function of teleost macrophages have been extensively studied (reviewed by Zelikoff et al., 1991; Secombes and Fletcher, 1992; Secombes, 1996). Macrophage modulation can occur following incubation with immunoregulatory molecules such as macrophage activating factor (MAF), tumor necrosis factor α (TNF α), transforming growth factor β (TGF β), β -glucans, growth hormone, and neurotransmitters (Secombes, 1996). In addition, respiratory burst activity is exhibited by teleost macrophages following engulfment of foreign material (Secombes, 1996).

Fish, along with other ectothermic vertebrates, possess pigmented macrophage aggregates (MAs) in many organ systems which contain hemosiderin, lipofuscin (ceroid), and/or melanin pigments in varying quantities depending upon the species (Panel 1A and B; reviewed by Wolke, 1992). The size, pigmentation, and number of MAs have been found to be sensitive to environmental changes (natural or anthropogenic); however, high inter-individual variation in MAs often precludes their use as biomarkers (Wolke, 1992). The immunological importance of MAs in fish is unknown (Wolke, 1992; Zapata et al., 1996).

Neutrophilic, eosinophilic, and basophilic granulocytes have been described for a variety of fish species (reviewed by Ainsworth, 1992). Typical functions attributed to mammalian PMN during inflammation have also been determined for fish neutrophilic granulocytes, such as phagocytosis, respiratory burst activity, and chemotaxis (Rowley et al., 1988; Ainsworth, 1992). Neutrophilic granulocytes of many species are the first immune cell type to migrate to the site of inflammation (Suzuki and Iida, 1992).

Many teleost species possess immune cells involved in spontaneous and non-specific cytotoxicity against transformed mammalian cells, parasites, and virally /bacterially-infected cells (reviewed by Evans and Jaso-Friedmann, 1992; Secombes, 1996). Because of morphological and functional differences from their mammalian counterparts, NK cells are generally termed non-specific cytotoxic cells (NCCs) in teleosts.

c. Adaptive Immunity

Teleost fish possess the genetic machinery necessary to mount both humoral and cell-mediated immune responses (reviewed by Kaattari and Piganelli, 1996; Manning and Nakanishi, 1996). This includes genes encoding RAGs (Willett et al., 1997), MHC class I and II (Stet et al., 1998), TCR (Partula et al., 1995; Zhou et al., 1997; Wilson et al., 1998; Haire et al., 2000; Wermenstam and Pilstrom, 2001), Ig heavy and light chains (Pilstrom and Bengten, 1996), cytokines (Secombes et al., 1999), and various immune co-receptors (Hansen and Strassburger, 2000; Park et al., 2001; Yoder et al., 2001). In addition, the functional equivalents of APCs are present in fish (Vallejo et al., 1991; 1992; Miller et al., 1994). Fish species have been found to exhibit: antigen-specific antibody production (Kaattari and Piganelli, 1996); CTL activity (Verlhac et al., 1990; Yoshida et al., 1995; Stuge et al., 2000); MLR (Miller et al., 1986); DHR (Manning and Nakanishi, 1996); graft-versus-host reactions (Nakanishi, 1994); and, xenograft /allograft rejection (Manning and Nakanishi, 1996).

Until recently, teleost antibody was thought to be of only the IgM isotype and exist as tetramers with a classical 2 heavy chain – 2 light chain subunit structure (reviewed by Kaattari and Piganelli, 1996). However, recent investigations in two teleost species have described a second isotype similar to mammalian IgD (Miller et al., 1998). Although it appears that fish possess limited isotypic diversity, several studies have indicated some heterogeneity of IgM heavy chains (Lobb and Olson, 1988; Sanchez et al., 1989) and isotypic variants of light chains (Lobb et al., 1984; Sanchez and Dominguez, 1991; Whittington, 1993). Several investigators have also

shown that subpopulations of teleost IgM exist which differ in their covalent structure (Lobb and Clem, 1981; 1983; Warr, 1983; Lobb, 1986; Whittington, 1993). The tetrameric molecules appear to contain various configurations in which the presence of both inter-heavy and inter-subunit disulfide bonds varied, producing the appearance of an array of protein bands from 90 to 750KD under non-reducing, denaturing conditions in SDS-PAGE (Kaattari and Piganelli, 1996).

The gene arrangement of teleost Ig heavy chain is similar to that described for mammals with variable (V_H), diversifying (D), and joining (J_H) regions upstream of constant μ and δ segments (Miller et al., 1998). The light chain gene organization is similar to that of elasmobranchs such that V_L , J_L , and C_L segments appear in multiple clusters (Miller et al., 1998). In addition, teleost fish B-cells demonstrate Ig heavy chain germline rearrangements and allelic exclusion (Miller et al., 1994).

The development of various teleost immune cell lines has greatly advanced our understanding of the humoral response in fish (Miller et al., 1998). B-lymphocytes of fish express both BCR and secreted forms of IgM. Cross-linking of BCR results in protein tyrosine phosphorylation, calcium ion influx, and B-cell proliferation (Van Ginkel et al., 1994). Elicitation of anti-hapten responses to T-cell independent antigen in catfish has been shown to require APCs and B-cells, but not T-cells (Miller et al., 1985). The requirement for APCs (i.e., macrophages) is due to IL-1 release from this particular cell population (Ortega, 1993). In addition, responses to T-cell dependent antigens appear to require the presence of both T- and B-cells along with APCs. The T-cell dependent response necessitates that antigen be processed by APCs prior to

elicitation of the response; the role that MHC molecules play in humoral immunity in fish has yet to be clearly-defined (Vallejo et al., 1990). Various effector mechanisms have been described for teleost antibody including: neutralization; opsonization; complement fixation; precipitation; and, agglutination (Kaattari and Piganelli, 1996). In addition, protective antibodies against bacterial and viral pathogens are produced following immunization. Although teleosts do not appear to possess a secondary humoral response as dramatic as that seen in mammals, secondary exposure of fish to sub-immunogenic doses of an antigen elicits a significantly greater response compared to that observed following the initial exposure (Arkoosh and Kaattari, 1991).

Characterization of specific cell-mediated cytotoxicity in teleost species had been hampered, until just a few years ago, due to a lack of defined cellular and molecular markers and *in vitro* systems (Stuge et al., 2000). Early studies of cell-mediated immunity in fish demonstrated that acute (less than 14 d) allograft rejections of skin and scales occur in many teleost species (Manning and Nakanishi, 1996). The cell-mediated phenomenon of graft-versus-host reaction (GVHR) has also been described in studies that employed triploid and tetraploid ginebuna-goldfish (*Carassius auratus*) hybrids (Nakanishi and Ototake, 1999). Finally, DHR responses have been recorded in response to bacterial and parasitic antigens in various species of fish (Manning and Nakanishi, 1996).

Recently, allospecific cytotoxic responses of fish leukocytes have been demonstrated *in vitro* (Fisher et al., 1998). In addition, intricate studies utilizing TNP-modified allogeneic and autologous cells have indicated that genetically-restricted

target cell recognition is involved in the specific CTL response of fish following both *in vitro* and *in vivo* priming (Verlhac et al., 1990; Hasegawa et al., 1999). The recent discovery of TCR alpha and beta chains sequences in fish (Zhou et al., 1997) has allowed for the demonstration of at least four sub-populations of cells involved in the CTL response of channel catfish (Stuge et al., 2000). One population was identified as TCR $\alpha\beta^+$ allospecific cytotoxic cells (i.e., equivalent to mammalian CTLs), while other groups include: TCR $\alpha\beta^+$ non-specific cytotoxic cells; TCR $\alpha\beta^-$ allospecific cytotoxic cells; and, TCR $\alpha\beta^-$ non-specific cytotoxic cells. In addition, a TCR $\alpha\beta^+$ cell sub-population that proliferated in response to allogeneic antigen, but possessed no cytotoxicity towards target cells was also observed. The TCR $\alpha\beta^+$ sub-population may represent a "T_H-like" cell population (Stuge et al., 2000).

5. Fish Immunotoxicology

Inherited immunodeficiency disease has not been described in fish species. However, many infectious agents apparently target fish immune organs and/or cells including infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV). Determination of the roles that viral-induced immunosuppression in fish might play in host resistance to subsequent infections is difficult. These diseases often result in acute mortalities due to disrupted osmoregulation via damaged kidney excretory tissue adjacent to hematopoietic tissue. Thus, conclusive data regarding the importance of the fish immune response in resistance to infectious disease is limited to studies using immunostimulatory agents

or vaccines. Various immunostimulants have been demonstrated to enhance fish immune functions (both innate and adaptive) and host resistance to infectious disease (reviewed by Sakai, 1999). For example, similar to what has been observed in mammals, exposure of fish to levamisole increases the number of peripheral blood leukocytes and levels of serum lysozyme, as well as enhances phagocyte function (Siwicki, 1987; 1989). Furthermore, levamisole injection increases the resistance of fish to various bacterial pathogens (Sakai, 1999). In addition, several effective vaccines that increase the host's humoral response to bacterial pathogens are currently used in aquaculture (reviewed by Kaattari and Piganelli, 1996; Gudding et al., 1999). With the exception of X-irradiation (Shechmeister et al., 1962), decreased host resistance to infectious agents following exposure of fish to mammalian immunosuppressants has not been well-studied; although altered immune function has been demonstrated following exposure of fish to cyclophosphamide, X-irradiation, and corticosteroids (reviewed by Zelikoff, 1994).

It is becoming increasingly evident that anthropogenic pollution may induce immunomodulation in organisms inhabiting aquatic environments (Ross et al., 1996a; 1996b; Luebke et al., 1997). However, associations between pollution, immune changes, and disease prevalence are difficult to substantiate due to the complex organization of aquatic ecosystems, unknown exposure histories, existing fishing pressure, and current trends in climatic change (Bucke, 1993; Vethaak and Jol, 1996; Austin, 1999; Harvell et al., 1999). Several diseases/lesions are commonly observed in fish from polluted waters such as epidermal papillomas, fin and tail rot, ulcerations,

lymphocystis, lymphosarcomas, and hepatocellular carcinomas. With the exception of non-viral tumors (Malins et al., 1988), it could be hypothesized that contaminant-induced immunosuppression may contribute to the high prevalence of some of these pathologies (Bucke, 1993).

Since fish and mammals appear to share some of the major components of innate and adaptive immunity, many of the immunotoxicological assays utilized in rodent studies have also been applied to fish models (Karol, 1998; Burns-Naas et al., 2001). Fish immunotoxicological models may serve two purposes in bridging the gap between human and ecological risk assessment (Zelikoff, 1998; Zelikoff et al., 2000; 2002). Although there is a growing body of evidence that a variety of xenobiotics can alter fish immune function (reviewed by Zelikoff, 1994; Anderson and Zeeman, 1995; Anderson, 1996), data utilizing comparable experimental protocols (i.e., chemical dose, exposure duration, exposure route) to those employed in rodent studies must be generated in order to better validate laboratory fish models as predictors of mammalian immunotoxicity. Furthermore, environmentally relevant exposure to xenobiotics (i.e., complex chemical mixtures) using fish immunotoxicological models may provide evidence for potentially detrimental effects upon feral fish populations (Bucke, 1993; Luebke et al., 1997). Since fish represent an extremely diverse group of organisms (> 25,000 species), it would be expected that they could serve as a nearly ideal alternate model for investigating immunotoxicology. In addition, through the use of fish models, valuable information could be gained regarding the phylogenetic conservation of responses to xenobiotics.

Mammalian immunotoxicological assays have been successfully adapted for use in a number of fish species including: tilapia (Holladay et al., 1998); rainbow trout (Cleland et al., 1988); chinook salmon (Arkoosh et al., 2001); mummichog (Faisal et al., 1991); turbot (Hutchinson et al., 1999); and, Japanese medaka (Beaman et al., 1999). Several immune assays, modified for use in fish species, have been described in detail (Stolen et al., 1990; 1992; 1994; Stolen and Fletcher, 1994) and include complement fixation, PFC; respiratory burst activity, phagocytosis, lymphocyte proliferation, MLR, allograft rejection, DHR, CTL, serum IgM ELISA, NCC activity, and host resistance challenge assays.

B. The Medaka Model

1. The Japanese Ricefish: Its Utility for Modern Biological Research

In the summer of 1994, the space shuttle *Columbia* lifted-off for a 2-week voyage into outer space. On board were four Japanese medaka (*Oryzias latipes*). Their mission, which they successfully completed, was to become the first vertebrates to mate and reproduce in space (Ijiri, 1995). This event was just one “first” in the long history of medaka biological research that spans nearly a century (Wittbrodt et al., 2002). Vertebrate Mendelian inheritance was first demonstrated using medaka in 1913 (Ishikawa, 1913; Toyama, 1916), and early medaka genetic studies revealed that cross over between the X and Y chromosomes occurs in species possessing backbones (Aida, 1921). The English translation of the word medaka is “tiny fish with big eyes” (Packer, 2001). The Japanese medaka, indigenous to Japan, Korea, Formosa, and

China, was originally known by the scientific name *Poecilia latipes* (Temminck and Schegel, 1846) which was later changed to *Oryzias latipes* by Jordan and Snyder (1906) to better describe its habitat, the rice (*Oryzias sativa*) fields of Eastern Asia. Hence, the common name for medaka in the Western world became the ricefish.

Modern studies in genetics have utilized medaka to study animal development (particularly the nervous system and eye), pigmentation, and sex determination (Wittbrodt et al., 2001). Over 100 mutant strains of medaka exist today (Ishikawa, 2000) including one strain that is totally transparent throughout life (Wakamatsu et al., 2001). Although applications of the medaka model to modern genetics research have lagged behind those of zebrafish (*Danio rerio*) and pufferfish (*Fugu rubripes*), medaka possess a number of advantages over both species including: (1) XY type sex determination; (2) availability of inbred lines (12 in total); (3) genetic information on wild populations; (4) active transposable elements; and, (5) embryonic stem (ES)-like cells (Ishikawa, 2000). The medaka has also become an important tool for studying transgenic animal production (Muir and Howard, 1999). A wealth of information concerning medaka biology is available online at the Medakafish Homepage maintained by investigators at Nagoya University, Japan (<http://biol1.bio.nagoya-u.ac.jp:8000>).

2. Medaka: A Model for Carcinogenesis & Toxicological Research

Various sub-disciplines of biology have turned to alternative animal models (i.e., *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster*, and *Caenorhabditis*

elegans) in order to address scientific, economical, and ethical problems. In toxicology and cancer research, the medaka model appears to be at the forefront of the alternative animal systems being studied. Classical toxicological and cancer investigations primarily utilized rodent species, and this is also true of current research. However, because of ethical concerns, there is increasing pressure from the public to use non-mammalian organisms. In addition, the discovery of detrimental effects of anthropogenic pollution upon aquatic ecosystems has gained much attention in the last few decades.

Advantages of medaka for toxicological studies include: small size (< 1 g body weight [BW]) permitting the accommodation of large numbers of animals in a limited space; resilient nature, allowing for its maintenance at high tank densities; transparent embryos which provide for identification of abnormalities during embryonic development; short generation time permitting multi-generational studies; easily bred under laboratory conditions; easily adaptable to a wide range of temperatures (10 – 40 °C) and salinities (fresh to brackish water); inexpensive to maintain; and, easily identifiable sexual characteristics as adults making the determination of males and females relatively simple (Yamamoto, 1975). Of particular interest for cancer research is the fact that medaka possess a very low incidence of spontaneous tumors compared to their rodent counterparts. In addition, medaka have been found to be highly sensitive to chemical-induced carcinogenesis with very short tumor induction times (~ 6 week; Boorman et al., 1997) In general, medaka have been utilized in numerous investigations to determine teratogenic, reproductive, and carcinogenic

effects of various compounds and environmental samples (Egami, 1955; Yamamoto, 1958; Ishikawa et al., 1975; Hoover et al., 1984; Hawkins et al., 1985; Wester and Canton, 1986; Cooper and McGeorge, 1991; Grey and Metcalfe, 1997; Kim and Cooper, 1998; Patyna et al., 1999).

3. *Medaka Immunology & Immunotoxicology*

In our laboratory, the medaka model is being specifically employed to investigate the effects of chemical and physical environmental stressors upon immune function and host resistance. The significance of this research is justification of the medaka as an alternate animal model for rodents in immunotoxicological studies, and as a biomarker for predicting anthropogenic stressors. Additionally, when compared to results obtained in rodent studies, information can be gained regarding evolutionarily conserved responses to xenobiotic exposure (comparative immunotoxicology).

A battery of immune assays has been developed in medaka for determination of xenobiotic-induced effects upon innate, cell-mediated, and humoral immunity. In addition, a medaka host resistance challenge assay has been validated using the bacterial pathogen *Yersinia ruckeri*. These assays have been found to be sensitive to the immunomodulatory effects of heavy metals, insecticides, PAHs, halogenated aromatic hydrocarbons (HAHs), physical stressors (i.e., hypoxia and temperature stress), and contaminated groundwater (Zelikoff et al., 1996; 2000; 2002; Luebke et al., 1997; Zelikoff, 1998; Beaman et al., 1999; Carlson et al., 2002; in press; Duffy et al., in press).

C. Benzo[a]pyrene (BaP)

1. Physical & Chemical Characteristics of BaP

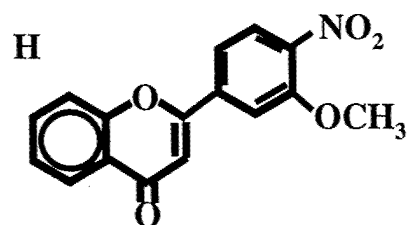
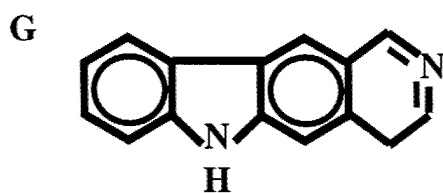
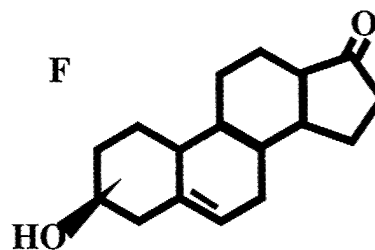
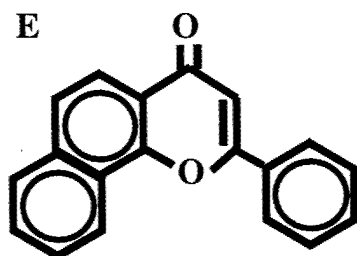
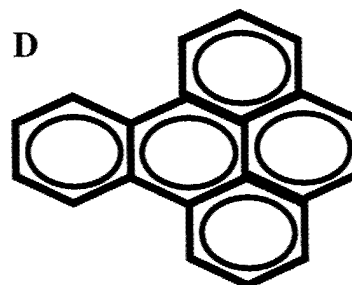
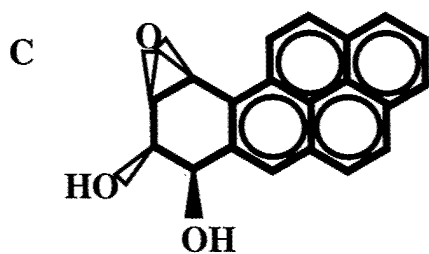
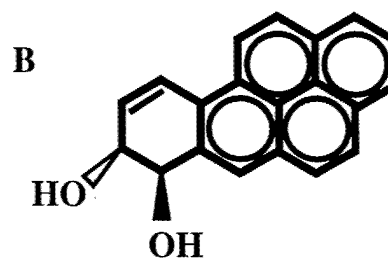
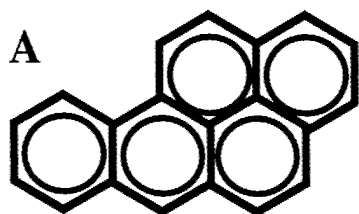
BaP is a polycyclic aromatic hydrocarbon (PAH) consisting of five connected aromatic benzene rings (Figure 1A; CAS Registry No.: 50-32-8). Synonyms of BaP include benzo[def]chrysene, 3,4-benzopyrene, 3,4-benzpyrene, and benz[a]pyrene (U.S. EPA, 1990). BaP in pure form (MW = 252.3 g/mole) exists as yellow crystalline needles or plates that fluoresce yellow-green in UV light. Although BaP is relatively insoluble in aqueous solvents (water solubility is 3.8×10^{-6} g/L), it is quite soluble in organic solvents such as benzene, toluene, acetone, and DMSO leading to a high octanol-water partition coefficient ($K_{ow} = 1.15 \times 10^6$).

2. Environmental Fate of BaP

BaP, along with many other PAHs, is formed whenever organic material is burned. Therefore, the production of BaP occurs by both anthropogenic (i.e., residential heating, automobiles, and industry) and natural (i.e., forest fires and volcanoes) processes. Incomplete combustion of fossil fuels (i.e., gasoline, diesel, oil, or coal) or biota, as well as tobacco smoke, leads to the release of BaP into the air. From the air, BaP is then distributed throughout the atmosphere and deposited into soil and water.

Residential heating is probably the largest individual source of BaP. Home heating primarily results in incomplete combustion of fuel and uncontrolled emissions.

Figure 1. Chemical structures of all test chemicals utilized in this study. A. Benzo[a]pyrene [BaP]. B. Benzo[a]pyrene-*trans*-7,8-dihydrodiol (+/-) [BD]. C. Benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (+/-)(anti) [BPDE]. D. Benzo[e]pyrene [BeP]. E. α -naphthoflavone [ANF]. F. dehydroepiandrosterone [DHEA]. G. ellipticine [ELP]. H. 3'-methoxy-4'-nitroflavone [MNF].



Currently, the largest contributor to residential emissions is woodburning at an estimated release of 72 metric tons per year in the U.S. alone (U.S. EPA, 1985). Historically, incomplete combustion of coal was the major source. Furthermore, high concentrations of atmospheric BaP are found in industrialized urban areas (0.2 to 19.3 ng/m³) compared to rural sites (0.1 to 1.2 ng/m³; Puckhat, 1981). The total release of BaP via combustion in the U.S. was estimated by the EPA (1985) as 154 metric tons or 90% of total BaP releases. High concentrations of BaP could also be expected in the air over BaP-contaminated hazardous waste sites. In addition, 98% of all BaP releases are thought to be directly into the air (EPA, 1990) with equal amounts of the remainder released directly onto land and water.

Due to the highly hydrophobic nature of BaP, atmospheric BaP is associated with particulate matter. Dispersion of particle-bound BaP is the primary transport mechanism in the air. It has been estimated that nearly half of atmospheric BaP is deposited to land and water by dry deposition with a much smaller percentage removed by wet deposition (EPA, 1985). Deposition of particles depends upon several factors including particle size and climatic conditions. Since BaP has a low vapor pressure (5.6×10^{-9} mmHg at 25 °C), once deposited from the air to water or land, it will remain there and eventually end up in the soil or sediment (EPA, 1990). Thus, vaporization of BaP from water or soil to air is highly unlikely, although particle-bound BaP could be aerolized into the atmosphere from contaminated soil or water. Atmospheric BaP can be relatively persistent since its presence has been detected in areas long distances from the source (EPA, 1990). Photochemical oxidation of

particulate BaP probably plays a major role in its fate, with atmospheric half-life estimates ranging in the hours (EPA, 1990). BaP absorbs solar radiation at wavelengths greater than 300 nm and singlet oxygen oxidizes the compound to create endoperoxides.

Besides dry deposition of atmospheric BaP (originally from natural or anthropogenic sources) into aquatic environments, BaP can be released into aqueous media via sewage/industrial effluents and accidental releases such as oil spills. BaP deposited into water is expected to partition into sediment or suspended particles. The small amount of BaP that is dissolved is subjected to direct photolysis with an estimated half-life of 1.2 h (Smith et al., 1978); high concentrations of oxidants such as ozone or chlorine will likely speed up the process (EPA, 1990). Dissolved BaP will also accumulate in aquatic biota; bioaccumulation depends upon the capacities of exposed organisms to metabolize BaP. Very low levels of BaP are expected in groundwater due to its propensity to bind strongly to soil and resist leaching. In addition, desorption of BaP from sediments to water is highly unlikely due to the hydrophobic nature of this compound. The Maximum Contaminant Level Goal (MCLG) for BaP is set at zero for drinking water (EPA, 1995). The enforceable standard for BaP (i.e., Maximum Contaminant Level [MCL]) has been set at 0.2 $\mu\text{g/L}$ due to difficulties in the detection of low levels of BaP.

Soil is a major deposition site for BaP and most likely represents a relative reservoir for accumulation of BaP and other hydrophobic PAHs. Biodegradation is the major fate of soil-borne BaP. However, this process is very slow and half-lives are

measured in years (EPA, 1990). Biodegradation can also be slowed down even further by the existence of other xenobiotics in contaminated soil that may be acutely-toxic to biodegrading microorganisms. BaP concentrations in soils usually fall into the range of 100 to 1,000 $\mu\text{g}/\text{kg}$, although a value as high as 650,000 $\mu\text{g}/\text{kg}$ has been recorded (Edwards, 1983). High BaP soil levels are most likely encountered near major industrial operations, large roadway intersections, sites of forest fires or volcanic activity, and creosote wood processing plants. Soil-associated BaP can be released into aquatic systems by erosion processes leading to deposition in sediments.

BaP in sediment is persistent and accumulates due to extremely slow biodegradation processes (EPA, 1990). Degradation is affected by multiple parameters such as redox state, temperature, sediment structure, and biological activity (Gardner et al., 1979). Herbes and Schwall (1978) reported that sediment highly-contaminated with PAHs possessed microbial populations unable to oxidize BaP. Likely sources of sediment BaP include; atmospheric fallout, erosion of contaminated soil, industrial effluents, urban runoff, accidental release (i.e., oil spills). Sites of higher anthropogenic activity (i.e., sewage release, manufacturing, shipping, and boating) possess higher sediment BaP levels as compared to more pristine sites. Elevated levels of BaP and other PAHs have been detected in contaminated sediments throughout North America including such areas as Eagle Harbor, WA (1350 $\mu\text{g}/\text{g}$ total PAH), Newark Bay, NJ (1960 $\mu\text{g}/\text{g}$ total PAH), the Black River, OH (1096 $\mu\text{g}/\text{g}$ total PAH), and the Elizabeth River, VA (21,200 $\mu\text{g}/\text{g}$ total PAH; Collier et al., 1986; Roberts et al., 1989; Baumann and Harshbarger, 1995; Huntley et al., 1995). Specifically, Catallo

and Gambrell (1987) reported a BaP concentration of 610 $\mu\text{g/g}$ in sediment taken from Bayou Bonfouca, LA. The aforementioned sediment sample was estimated to be approximately 25% creosote. Release of BaP from sediment into the water column is nominal. However, sediment disruption can lead to the suspension and transport of particulate BaP. The removal of sediment BaP by aquatic organisms is dependent upon many factors. Uptake of sediment BaP is likely in aquatic food chains that possess benthic organisms efficient in accumulating BaP without significant metabolism. Marine and freshwater organisms vary tremendously in both their affinity for removing sediment BaP and metabolic activity towards BaP (Meador et al., 1995). Considerable variation can even be observed between closely related species.

3. Uptake, Distribution, Metabolism, and Elimination of BaP in Rodent and Fish Studies

BaP is rapidly absorbed by inhalation, oral, and dermal exposure routes in rodent models (reviewed by EPA, 1990). Following inhalation of BaP adsorbed onto particles, uptake is dependent upon both BaP deposition in the lungs and mucociliary clearance, as well as subsequent absorption in the gastrointestinal tract (Sun et al., 1982). Following oral exposure, BaP passes to the small intestine where it is solubilized by bile salts prior to absorption (Laher and Barrowman, 1983). In addition, rats housed with PAH-contaminated soil (21 $\mu\text{g BaP/g soil}$) for 3 d accumulated BaP in the liver and lungs at concentrations of 69 and 648 ng/g of fresh organ, respectively (Fouchecourt et al., 2001).

Absorption of BaP by fish occurs through the gastrointestinal tract, gills, and skin (Meador et al., 1995). Balk et al. (1984) demonstrated that Northern pike (*Esox lucius*) absorb BaP via the stomach and intestine following exposure to fish muscle contaminated with [³H]BaP (7.5 µg /g). Exposure of pike to waterborne BaP (75 ng/L) resulted in rapid uptake via the gills and to a lesser extent, via the skin, fins, nares, and lining of the oral cavity (Balk et al., 1984). Zebrafish (*Brachydanio rerio*) exposed to sediment contaminated with BaP (2.5 µg /g sediment) exhibited a rapid uptake that peaked around 250 ng BaP /g BW at 48 h (Djomo et al., 1996).

Once absorbed, BaP disseminates rapidly to many organs /tissues in rodent models. However, the relative concentration of unmetabolized BaP present in any one organ is dependent upon the route of administration and metabolism (EPA, 1990). For example, 5 min following intratracheal instillation of BaP, most of the absorbed dose was found in the lungs, followed by the liver, blood, and intestines (Weyland and Bevan, 1986). At 6 h post-instillation, the highest concentrations of BaP were present in the intestines, lungs, liver, and blood. In contrast, 6 h following oral gavage of rats, high levels of unmetabolized BaP were present in the blood, with much lower levels in the liver and lungs (Ramesh et al., 2001).

A similar rapid distribution has also been demonstrated in fish exposed to waterborne or dietary BaP. One day following dietary BaP exposure, pike exhibited significant levels of BaP in the liver, posterior kidney, and intestine (Balk et al., 1984). Pike exposed to waterborne BaP demonstrated a rapid accumulation of BaP in several

organs that plateaued at around 18 h, while concentrations in the kidney, liver, bile and gall bladder, and eye lens continued to rise for several days (Balk et al., 1984).

In descending order, the gills, followed by the bile and gall bladder, liver, and muscle, accumulated the highest concentrations of BaP 3 h following waterborne exposure of pike.

Once in the body, BaP rapidly enters most cell types by crossing the plasma membrane and incorporating into cytoplasmic membranes within several minutes (Barhoumi et al., 2000). Several microsomal enzymes have been implicated in the initial metabolism (Phase I biotransformation) of BaP in mammals including CYP1A1 and CYP1B1. These enzymes catalyze the formation of arene oxides at the 4,5-, 7,8-, or 9,10-positions. Arene oxides of BaP can spontaneously form phenols or become conjugated to glutathione (i.e., Phase II biotransformation). Glutathione-BaP conjugates are excreted, while phenols can be further oxidized into reactive quinones. Microsomal epoxide hydrolase catalyzes the formation of *trans*-dihydrodiols from BaP arene oxides. BaP-dihydrodiols can be conjugated by glucuronidation or sulfonation by Phase II biotransformation and excreted. Dihydrodiol dehydrogenases catalyze the formation of reactive catechols from BaP-dihydrodiols which undergo "redox cycling" prior to the formation of BaP-*o*-quinones. BaP-dihydrodiols can also be metabolized by cytochrome P450s (i.e., CYP1A1, CYP1B1) to form highly reactive diol epoxides. These BaP-diol epoxides can become covalently attached to glutathione (and subsequently eliminated), spontaneously form tetraols, or react with cellular proteins and nucleic acids. Reactive diol epoxides are readily created from the

metabolism of BaP-7,8-dihydrodiol (BD; Figure 1B), by the cytochrome P450 (i.e., CYP1A1 and CYP1B1) or prostaglandin synthetase systems, to form BaP-7,8-dihydrodiol-9,10-epoxide (BPDE; Figure 1C); BPDE is thought to be the major carcinogenic metabolite of BaP.

Thus, Phase II conjugation of BaP by UDP-glucuronyl transferase, sulfate transferase, and glutathione transferase can be considered detoxification pathways leading to the eventual excretion of BaP. In contrast, Phase I biotransformation can lead to bioactivation of BaP by forming reactive metabolites that interfere with cellular macromolecules, thus, impeding its elimination. Basal metabolic activity towards BaP varies considerably among cell types, tissues, organs, individuals, strains, and species of mammals.

The parent compound of BaP also binds specifically to several proteins in the cell cytoplasm. It has been well characterized in mammals that BaP binds to the unliganded AhR complex that consists of the AhR, AhR-interacting protein (AIP), and two heat shock protein 90 (HSP90) molecules (Meyer and Perdew, 1999). BaP-AhR binding causes HSP90 and AIP to dissociate from the complex; AhR-BaP is then translocated to the nucleus. Once in the nucleus, the AhR complexes with the AhR nuclear translocator (ARNT) protein to form a heterodimer. In mammals, the ligand-AhR-ARNT complex can then bind to xenobiotic response elements (XREs; also termed dioxin response elements or DREs) upstream of various genes (termed the AhR gene battery) such as CYP1A1, CYP1A2, CYP1B1, aldehyde dehydrogenase 3, and glutathione transferase YA (Nebert et al., 2000). XRE binding by AhR-ARNT

complexes can result in the transcriptional enhancement of these genes (Henry et al., 1997). Thus, this BaP-activated signal transduction pathway regulates the expression of the very same enzymes (i.e., CYP1A1, CYP1B1) that are responsible for BaP metabolism. There is also considerable variation in the inducibility of AhR pathways following BaP exposure between different cell types, organs, and species.

Many of the same components involved in BaP metabolism of mammals have also been described in fish species (reviewed by Stegeman and Hahn, 1994). Fish enzymes of the CYP1A family possess structural and functional properties similar those of mammalian CYP1A1; however, they also possess properties of mammalian CYP1A2 (Stegeman, 2000). Since this enzyme appears to be ancestral to both mammalian sub-types, it is generally termed "CYP1A." In addition, two CYP1A genes have been described for rainbow trout that were found not to be orthologous to mammalian CYP1A1 and CYP1A2 (Rabergh et al., 2000). Recently, fish CYP1Bs have been cloned in plaice (*Pleuronectes platessa*) and scup (*Stenotomus chrysops*) (Godard et al., 2000; Leaver and George, 2000).

Both AhR and ARNT genes have also been cloned in teleost fish. Two AhR genes have been identified in the mummichog (i.e., AhR1 and AhR2) that demonstrate differences in tissue expression (Karchner et al., 1999). Furthermore, XREs have been found upstream of the fish CYP1A and CYP1B genes. Increased binding to fish- and mouse-derived XRE-containing oligonucleotides following exposure to TCDD, PCBs, and BNF has been demonstrated (Roy and Wirgin, 1997; Karchner et al., 1999). Generally, exposure of fish or fish cells to BaP leads to the up-regulation of CYP1A

expression (Stegeman and Hahn, 1994). However, as shown in mammals, considerable variation exists in both basal and BaP-induced metabolic activities among various fish species, and between different cell types and organs within species.

For mammals, it is generally agreed that BaP must be metabolized prior to its elimination from the body, and this metabolism is the rate-determining step in BaP excretion (EPA, 1990). Excretion occurs mainly through biliary transport to the intestinal tract and removal in the feces, although BaP metabolites can be detected in urine. Time to elimination by different routes of administration is as follows: dermal > lung > oral (EPA, 1990).

Elimination of BaP in fish follows a similar pattern as that described for mammals. However, BaP excretion in the urine is likely to depend upon the extent of urine formation since freshwater fish produce far greater amounts of urine compared to those in salt water. Furthermore, the fish gill does possess some metabolic activity, thus providing an additional pathway for excretion. Sea bass (*Dicentrarchus labrax*) injected IP with BaP exhibited a BaP half-life of 2.4 days (Lemaire et al., 1990). A BaP half-life of 5.75 days was reported during depuration of zebrafish exposed for 24 h to BaP-contaminated sediment (Djomo et al., 1996).

4. Toxicity & Carcinogenicity of BaP

Although there are no reports of acute BaP-induced lethality following exposure of rodents via dermal, oral, or inhalation routes, mortality and decreased

longevity has been described following subchronic BaP oral exposure and IP injection of mice (Robinson et al., 1975). Interestingly, Robinson et al. (1975) only demonstrated BaP-induced mortality in Ah "non-responsive" mice, probably due to decreased BaP detoxification and elimination in this particular mutant mouse strain (longer persistence). In fact, mice lacking the CYP1A1 gene (*Cyp1a1*^{-/-}) possess a significantly slower clearance rate for blood-borne BaP resulting in higher level hepatic BaP DNA adducts compared to *Cyp1a1*^{+/-} mice (Uno et al., 2001). Production of BaP DNA adducts in *Cyp1a1*^{-/-} mice was probably due to CYP1B1 or prostaglandin synthetase activity.

In the earlier rodent studies, BaP-induced death was attributed to bone marrow depression and subsequent aplastic anemia, pancytopenia, hemorrhage, and infection (Robinson et al., 1975). Reduced bone marrow cellularity has been demonstrated following 5 daily IP injections of 100 µg BaP /g BW in B6C3F1 mice (Holladay and Smith, 1995). In these studies, decreased bone marrow cellularity was associated with decreased numbers of pro-lymphocytes. The sensitivity of pre-B lymphocytes in murine bone marrow cultures to BaP-induced apoptosis was inhibited by co-incubation with ANF or galangin (a dietary bioflavonoid and AhR antagonist), suggesting a role for the AhR in the observed toxicity (Quadri et al., 2000). Further studies revealed that BaP-induced pre-B cell apoptosis was dependent upon AhR expression in bone marrow stromal cells and not within the pre-B cells themselves (Near et al., 1999). Investigations of 7,12-dimethylbenz[a]anthracene (DMBA)-induced pre-B cell apoptosis have demonstrated that CYP1A1 activity has little to do

with the observed toxicity, and the most probable mechanism requires either CYP1A1-independent metabolism of PAHs or AhR signaling in stromal cells (Mann et al., 1999).

Systemic toxicities of BaP in mammals, other than hematopoietic toxicity, include dermal, reproductive, immunological, and developmental (teratogenesis) effects (EPA, 1990). Furthermore, BaP has been demonstrated to be genotoxic (mutagenic) in rodent cells. The genotoxicity of BaP is dependent upon metabolism to reactive intermediates which bind to DNA, forming bulky DNA adducts (EPA, 1990). Such adducts promote mutations and clastogenesis. In addition, BaP is a potent carcinogen in rodents via most exposure routes. The carcinogenesis of BaP is well-characterized and the major pathway involves cytochrome P450-mediated metabolism of BaP to BD and, ultimately to BPDE. Unrepaired adducts which survive cell division and result in important mutations of genes that regulate differentiation and proliferation may ultimately lead to malignancy. In general, tissues with proliferating cells are most susceptible to BaP-induced tumor formation such as the skin or lungs.

5. BaP-Induced Immunotoxicity

Early investigations into the immunotoxic potential of PAHs began with the hypothesis that chemical carcinogens suppress the immune surveillance of antigenic tumors. It was believed that the immune system played an important role in the "selection" and removal of neoplastic cells induced by viruses (Stjernsward, 1969). Furthermore, thymectomy of newborn mice resulted in an increased frequency of BaP-

induced sarcomas, stretching the theory to include chemical-induced carcinogenesis (Miller et al., 1963). However, a paradox arose since tumors induced by PAHs were found to be highly antigenic and rapidly rejected by syngeneic mice, but spontaneous tumors of a similar nature appeared to be relatively non-antigenic (Stjernsward, 1969). These observations led to the discovery that intramuscular injection of carcinogenic PAHs (3-methylcholanthrene [3MC], DMBA, and BaP) resulted in a reduction of antibody-forming cell numbers (Malmgren et al., 1952; Stjernsward, 1966). Thus, although it appears that PAH-induced tumors elicit a robust immune response in naive animals, immunotoxicity may allow for the same tumors to grow uninhibited in PAH-exposed animals. These studies also helped explain the possible mechanism behind observations of earlier studies that non-tumorigenic doses of BaP increased spontaneous viral tumor frequencies in mice (DeMayer-Guignard and DeMayer, 1965). In addition, PAHs that do not induce cancer (such as BeP and anthracene) failed to suppress immune function (Stjernsward, 1966), further indicating a link between PAH carcinogenicity and immunotoxicity.

Since the time of Stjernsward (1969), numerous investigators have demonstrated carcinogenic PAH-induced suppression of splenic PFC numbers using various exposure routes (i.e., intramuscular, subcutaneous, intraperitoneal, intratracheal, intragastric, *in utero*, and *in vitro*) in rodent models (reviewed by Ward et al., 1985; White et al., 1994). Suppression of the primary (IgM) T-cell dependent antibody response appears to be the hallmark of BaP-induced immunotoxicity in rodents; effects are not associated with decreased splenocyte viability. Specifically,

multiple subcutaneous BaP injections of B6C3F1 mice at cumulative doses as low as 40 µg /g BW resulted in significantly reduced splenic PFC responses following *in vivo* (Dean et al., 1983) or *in vitro* (Blanton et al., 1988) SRBC immunization. Fisher 344 rats exposed to BaP using an identical protocol demonstrated reduced PFC numbers (following *in vivo* SRBC immunization) at 100 µg /g cumulative doses (Temple et al., 1993). Additionally, age-related effects of BaP exposure upon humoral immune responses of B6C3F1 mice have been investigated (Lyte and Bick, 1985). These studies reported a greater reduction of PFC numbers following IP injection of BaP in aged (i.e., 23-26 months) mice as compared to relatively young adults (3-6 months) mice.

BaP-induced suppression of humoral immunity has also been observed following exposure via other routes. For example, exposure via a single oral gavage of C57BL/6J mice to 40 µg BaP /g BW produced a 40% suppression in splenic PFC numbers compared to vehicle control mice (Silkworth et al., 1995). In another study, seven daily IT instillations of 40 µg BaP /g in B6C3F1 mice resulted in significantly depressed PFC numbers in the spleen and lung-associated lymph nodes (LALN) following IP and IT SRBC immunizations, respectively (Schnizlein et al, 1987). Interestingly, Schnizlein et al. (1987) also reported significantly increased LALN PFC numbers in the same mice following IP SRBC immunization. This suggests the importance of the immunization route of the antigen in the observed immunotoxicity. Suppression of splenic PFC numbers following IT exposure to BaP has also been demonstrated in hamsters (Zwilling, 1977). In addition, *in utero* exposure of mice to

BaP (via a single maternal IP injection of 150 µg BaP /g BW at mid-gestation) resulted in decreased PFC numbers in the progeny that persisted for up 18 months after birth (Urso and Gengozian, 1980; 1982; 1984).

In vitro exposure of rodent splenocytes to BaP has been shown to directly affect immune cell function. Murine splenocyte cultures incubated with BaP at concentrations as low as 0.1 nM exhibited significantly reduced PFC numbers following *in vitro* SRBC immunizations (White and Holsapple, 1984; Lyte and Bick, 1985; Blanton et al., 1986; Urso et al., 1986; Kawabata and White, 1989; Tomar et al., 1991). BaP-induced cytotoxicity was only observed in splenocyte cultures receiving doses that were well-above those which suppressed immune function (i.e., >10µM; Urso et al., 1986). In addition, splenocytes from aged mice (23-26 months) appeared to be more sensitive to *in vitro* BaP exposure than those of younger mice (Lyte and Bick, 1985). Results from the aforementioned *in vitro* studies suggest that parent BaP compound can affect the function of immune cells in exposed hosts, likely ruling out the importance of immune mediators released from distant sites (i.e., liver).

Evidence for the suppressive effects of BaP exposure upon humoral immunity has also been provided by endpoints other than primary (IgM) PFC assays. Serum IgM specific for SRBCs was reduced after BaP exposure of both B6C3F1 mice and F344 rats (Temple et al., 1993). Dean et al. (1983) also demonstrated reduced secondary antibody (IgG) responses to SRBCs following subcutaneous injection of BaP at a cumulative dose of 200 µg /g BW. In the same studies, only the highest cumulative BaP dose (400 µg /g BW) significantly reduced LPS-stimulated B lymphocyte

proliferation (Dean et al., 1983); however, no BaP-induced alterations in the percentage of isolated splenic B-cells were observed.

Reduced PFC numbers following exposure to certain HAHs, such as PCBs, PCDFs, and polychlorinated dibenzodioxins (PCDDs), have been demonstrated in rodent models (reviewed by Burns-Naas et al., 2001). Humoral immunotoxicity induced by the aforementioned HAHs appears to segregate with the *Ah* locus (Vecci et al., 1983) and a clear structure-activity relationship (SAR) exists between the ability of HAHs to bind the AhR and induce immunotoxicity (Davis and Safe, 1988; Sulentic et al., 2000). Furthermore, AhR knockout mice appear insensitive to the immunotoxic effects of TCDD upon humoral immunity (Vorderstrasse et al., 2001).

It is well-known that carcinogenic PAHs activate the AhR pathway, leading to the up-regulation of CYP1A expression and subsequent CYP1A-mediated metabolism of parent PAHs into reactive, carcinogenic epoxides (Brooks and Lawley, 1964; Gelboin, 1969; Myer and Perdew, 1999; Nebert et al., 2000). Although such molecular mechanisms were unknown 30 years ago, researchers were aware of SARs existing between the carcinogenic and immunotoxic potentials of PAHs in rodent models (Stjernsward, 1969). Stjernsward (1966) described suppression of PFC responses following exposure to the carcinogens 3MC, DMBA, and BaP. Exposure to the relatively weaker carcinogen 7-methyldibenzanthracene (MDBA) gave intermediate immunosuppression, and the non-carcinogens BeP and anthracene failed to effect PFC numbers.

More recent investigations also failed to observe suppression of PFC numbers following subcutaneous injection of BeP at doses as high as 400 µg /g BW (Dean et al., 1983; White and Holsapple, 1984). Zwilling (1977) demonstrated that IT-instillation of BeP, at doses in which BaP proved immunotoxic, failed to alter PFC numbers in hamsters. Blanton et al. (1986) examined the effects of *in vitro* exposure to BeP and observed that a 1000 times greater concentration of BeP (7.93 µM) than BaP (7.93 nM) was necessary to significantly suppress PFC numbers. In contrast, Urso et al. (1986) reported that BeP concentrations as high as 100 µM failed to alter *in vitro* PFC numbers. BeP is structurally similar to BaP (c.f. Figure 1D); however, it is a weak AhR ligand at best (1 µM BeP displaces 7% of 10 nM [³H]TCDD bound to AhR compared to 96% for 1 µM BaP), leading to minimal induction of CYP1A enzymes (Bigelow and Nebert, 1982). In addition, BeP is poorly metabolized and fails to produce macromolecular binding metabolites (MacLeod et al., 1979; 1980). Although there appears to be a clear association between PAH carcinogenicity and immunotoxicity, studies using BeP have provided little information regarding possible molecular mechanisms underlying BaP-induced immunosuppression (i.e., AhR signaling or metabolite activity). However, such studies likely do rule out a direct effect of BaP (parent compound) upon immune cell function since one would expect that two such closely related compounds would alter biological membranes, proteins, and nucleic acids in an identical manner. In addition, BaP-induced immune suppression as a result of BaP binding to 8S and 4S cytoplasmic proteins is unlikely

since BeP has affinity for these receptors equal to that of BaP (Lesca et al., 1993; Sterling et al., 1994).

Initial studies investigating the role of the AhR pathway in BaP-induced immunotoxicity demonstrated that Ah non-responsive mice (DBA/2) were actually more sensitive to BaP-induced suppression of PFC number than the Ah responsive strain (B6C3F1) (White et al., 1985). However, BaP doses utilized in this study were significantly above those at which strain differences might exist (Silkworth et al., 1995). More recent investigations have utilized Ah responsive (C57BL/6J) and non-responsive (B6.D2) mice strains congenic at the *Ah* locus to study BaP-induced immunotoxicity at doses that reveal clear differences in BaP metabolism between the two strains (Silkworth et al., 1995). The latter studies reported that B6.D2 mice were one-fourth as sensitive to BaP-induced suppression of PFC numbers as the C57BL/6J strain.

In vitro studies have also investigated the role of BaP metabolism in BaP-induced suppression of PFC numbers. *In vitro* PFC numbers were significantly suppressed following exposure of mouse splenocytes to BD and BPDE at 1.0 and 10 nM, respectively (Kawabata and White, 1987; 1989). In addition, co-incubation of splenocyte cultures with 20 μ M α -naphthoflavone (ANF; an AhR antagonist and CYP1A1 inhibitor; c.f. Figure 1E) in combination with 2 μ M of either BaP or BD resulted in PFC numbers no different from those of cultures exposed only to vehicle (Kawabata and White, 1989). Thus, inhibition of BaP or BD metabolism appears to ameliorate observed immunotoxicity, indicating that the metabolites of BaP may be

the active immunotoxicants. These data help to further explain the association between PAH immunotoxicity and carcinogenicity since both the proximate and ultimate carcinogenic metabolites of BaP (i.e., BD and BPDE, respectively) have also been found to suppress humoral immunity. Data also exist that BaP metabolites other than BD and BPDE may be involved in immunosuppression such as the 3-hydroxy, 4,5-epoxide, and 6,12-dione metabolites (Kawabata and White, 1987; Tomar et al., 1991).

Since BaP metabolites suppress the PFC response, several investigations have been conducted to better characterize splenic metabolic activity. Incubation of splenocytes and splenic microsomes from untreated mice with [³H]BaP significantly increased the production of aqueous BaP metabolites (Kawabata and White, 1987). However, overall basal metabolic activity of mouse splenic microsomes has been shown to be 40-fold lower than that of hepatic microsomes (Ginsberg et al., 1989). BaP metabolism by the spleen is inhibited by incubation with ANF *in vitro*, suggesting the involvement of CYP1A1 (Kawabata and White, 1987). Further analysis of the BaP metabolites produced by exposure of splenic microsomes from untreated mice to [³H]BaP demonstrated that a relatively large percentage of BD was generated (Kawabata and White, 1989). In the same studies, incubation of splenic microsomes with [³H]BaP and ANF or trichloropropylene oxide (TCPO) resulted in no BD, indicating the need for splenic CYP1A1 and epoxide hydrolase in the production of immunotoxic BaP metabolites. Furthermore, splenic microsomes prepared from mice exposed to BaP (200 µg /g BW) produced significantly higher levels of BD *in vitro* compared to that from microsomes collected from vehicle-exposed animals (Kawabata

and White, 1989). Studies investigating the molecular basis for up-regulation of BaP metabolism revealed that murine splenocytes express both AhR and ARNT proteins and exposure to AhR ligands induced AhR binding to DREs, presumably leading to transcription and translation of CYP1A1 enzymes (Williams et al., 1996).

Detailed studies by Ladics et al. (1992a; 1992b; 1992c) determined that the macrophage was the cell type most capable of metabolizing BaP within the spleen, and that this cell population could generate BPDE from BaP via CYP1A-dependent and -independent (i.e., peroxyl radical) pathways. Other splenic cell types, including T-cells, B-cells, and PMN, were found not to significantly contribute to splenic BaP metabolism. Studies in F344 rats revealed that splenic macrophages express basal levels of AhR and AhR ligand-inducible levels of CYP1A protein and activity (Germolec et al., 1995). Immunohistochemical analysis of untreated rat spleen indicated that splenic macrophages express relatively high levels of AhR while lymphocytes exhibit weak AhR staining (Germolec et al., 1996). In contrast to the aforementioned findings, splenic and peripheral blood lymphocytes derived from rodents and humans have been found to possess inducible BaP metabolic activity following exposure to AhR ligands and mitogens (Whitlock et al., 1972; Suolinna et al., 1982; Wojdani and Alfred, 1984; Fung et al., 1999). Specifically, stimulation of splenic B lymphocytes with the mitogen LPS results in the up-regulation of AhR, ARNT, and CYP1A1 expression in the absence of AhR ligand in mice (Marcus et al., 1998). In addition, Lawrence et al. (1996) demonstrated that TCDD-induced AhR activation in murine T-cell lines was dependent upon the cell activation state. There is

even evidence for a human B-cell specific AhR complex possibly resulting from additional complex proteins or structural AhR variants present within these cells (Masten and Shiverick, 1996).

Since the T-dependent PFC response involves cooperation between splenic B-cells, T helper cells, and macrophages, several investigations have sought to determine which particular cell type was compromised following BaP exposure. *In vitro* studies determined that suppression of PFC numbers was most apparent when BaP was added to cultures close to the time of initial antigen exposure; addition of BaP to cultures 3 d following SRBC addition resulted in no immunosuppression (Blanton et al., 1986). Evidence for the early requirement of PAH exposure in the suppression of PFC numbers *in vivo* was demonstrated over 30 years ago (Stjernsward, 1969). In addition, *in vitro* SRBC immunization of splenocyte cultures following *in vivo* BaP exposure results in immunosuppression comparable to that observed following *in vivo* immunization (Dean et al., 1983; Blanton et al., 1988). Thus, the early temporal requirement of BaP for suppression of antibody formation suggests the importance of alterations in antigen presentation or APC-lymphocyte interactions and not direct effects upon anti-SRBC antibody producing B-cells (White et al., 1994). Evidence regarding the lack of BaP-induced effects upon immature B-lymphocytes is provided by the fact that BaP exposure (at non-cytotoxic doses) administered either *in vivo* and *in vitro* failed to alter T-independent polyclonal antibody responses to TNP-LPS (Dean et al., 1983; White and Holsapple, 1984; Ginsberg et al., 1989; Ladics et al., 1992c). Polyclonal antibody production to LPS does not require the presence of

macrophages or helper T-cells. Furthermore, *in vivo* and *in vitro* exposure to BaP resulted in significant suppression of PFC numbers in response to the T-independent antigen DNP-Ficoll (White and Holsapple, 1984; Ladics et al., 1992c). Antibody production following DNP-Ficoll immunization requires the active participation of both macrophages and B-cells, but not helper T-cells.

By the utilization of separation-reconstitution PFC techniques, investigators have reported that adherent (i.e., macrophages) splenocyte populations were affected by *in vivo* BaP exposure and non-adherent (i.e., B- and T-cells) populations were only minimally affected (Blanton et al., 1988; Ladics et al., 1992c). Meyers et al. (1987) demonstrated that BaP-exposed macrophages exhibited a significantly reduced capability to present the T-dependent antigen KLH to untreated T-cells, presumably due to decreased antigen uptake. In addition, Lyte and Bick (1986) showed that addition of IL-1 to splenocyte cultures resulted in amelioration of BaP suppressive effects upon *in vitro* PFC responses. Although studies by Myers et al. (1987) failed to detect alterations in BaP-exposed macrophage IL-1 secretion following Con A-elicitation, Ragg et al. (1997) demonstrated that dendritic cells exposed to BaP *in vivo* and antigen *in vitro* exhibited reduced IL-1 β secretion. Interleukin-1 is essential for proliferation and expression of IL-2 receptors in CD4⁺ T-cells; therefore, a deficiency in IL-1 could result in significantly reduced T-dependent antibody production. Additional studies reported that *in vivo* and *in vitro* BaP exposure decreases the ability of treated T-cells to respond to IL-2, as seen by decreased IL-2 secretion, IL-2 receptor expression, and IL-2 -induced proliferation (Lyte et al., 1987; Myers et al.,

1988). Furthermore, co-incubation of BaP-treated T-cells with untreated macrophages and recombinant IL-2 resulted in normal IL-2 responsiveness, suggesting that BaP-exposed macrophages failed to provide the proper signals (i.e., IL-1) required for T-cell activation (Myers et al., 1988).

There is also some evidence that BaP and BaP metabolites can directly affect lymphocyte function. Studies by Blanton et al. (1988) demonstrated that BaP-exposed B- and T-cells were functionally impaired when co-cultured with untreated peritoneal exudate macrophages. Investigations revealed that BaP and DMBA can alter lymphocyte signal transduction pathways (reviewed by Davila et al., 1995; Burchiel and Luster, 2001). Earlier studies have indicated that high concentrations of BaP (10 μ M) resulted in rapid (within 3 min) elevation of intracellular Ca^{++} levels in a human T-cell line (Krieger et al., 1994). These same studies demonstrated that this increase was probably due to increased activation of protein tyrosine kinases (PTKs). Altered intracellular calcium levels could lead to disruption of antigen-triggered signal transduction and, eventually, lymphocyte anergy / apoptosis. Mounho et al. (1997) determined that BaP, but not BeP, induced calcium elevation in human B- , T-cells, and monocytes isolated from peripheral blood. In these same studies, both BD and BPDE (3 μ M) were found to elevate T- and B-cell calcium levels to a greater extent than BaP (10 μ M). This suggests a role for BaP metabolism in the observed alterations. Exposure of human B-cell lines to ANF prior to incubation with BD or BPDE inhibited the observed calcium rise following BD exposure but not following incubation with BPDE; the data indicates that BPDE may be the ultimate metabolite

responsible for the observed alteration in calcium levels (Mounho and Burchiel, 1998). Since both BD and BPDE can increase phosphorylation of the PTKs Lyn and Syk, and inhibition of phosphorylation prevented BaP metabolite-induced calcium elevation, it appears that BaP metabolites could act upon protein tyrosine phosphatases (PTPs; Mounho and Burchiel, 1998).

Evidence also exists that BaP quinones (BPQs) may play a role in altered lymphocyte signaling and/or BaP immunotoxicity (Burchiel and Luster, 2001). Although it is not clear whether lymphocytes can produce BPQs by themselves, other splenocytes (i.e., macrophages) do possess peroxidase activity (Zhu and Trush, 1995). In addition, BaP metabolites have been shown to induce oxidative stress in human and mouse lymphocytes, resulting in glutathione depletion (Yuan et al., 1994; Romero et al., 1997). BPQs can form reactive oxygen species (ROS) by redox cycling or disruption of mitochondrial integrity, and it is speculated that ROS production could lead to inactivation of PTP and, ultimately, calcium elevation (Burchiel and Luster, 2001). Recent studies have indicated that BPQs (specifically BaP-7,8-dione) can alter calcium levels by disruption of ryanodine receptors (Pessah et al., 2001). BaP-7,8-dione is produced via oxidation of BD by dihydrodiol dehydrogenase. Since ryanodine receptors are expressed in lymphocytes (Hosoi et al., 2001), BPQ-induced inactivation of these receptors presents a viable partial explanation for elevated calcium levels. Despite the aforementioned evidence that BaP and BaP metabolites alter lymphocyte signaling, Burchiel and Luster (2001) conclude that these changes may have little to do with BaP-induced suppression of humoral immunity since the high levels of BaP

required for these responses actually result in apoptosis of human B-cells (Salas and Burchiel, 1998).

Although there appears to be overwhelming data that BaP affects humoral immunity, there is contradictory data concerning the effects of BaP upon cell-mediated immunity. Dean et al. (1983) reported significantly decreased T-cell proliferative responses following *in vivo* BaP exposure, while other investigators have demonstrated little effect of BaP upon T-cell responses to PHA (Wojdani and Alfred, 1984). Similarly, contradictory results for T-cell proliferation have also been reported following *in vitro* BaP exposure of murine splenocytes (Thurmond et al., 1988; Tomar et al., 1991). In addition, *in vitro* incubation of splenocytes with BaP results in decreased MLR, while *in vivo* exposure of mice failed to alter either this response, allograft rejection times, or delayed-type hypersensitivity to KLH (Dean et al., 1983; Thurmond et al., 1988). Interestingly, human T-cells appear to be highly sensitive to the anti-proliferative effects of BaP, with doses as low as 10 nM significantly inhibiting [³H]-thymidine incorporation (Mudzinski, 1993). Exposure to ANF and BaP resulted in amelioration of BaP-induced suppression of T-cell mitogenesis, suggesting a role for BaP metabolism in the observed alterations (Mudzinski et al. 1993, Davila et al., 1996).

Very little information exists regarding the effects of BaP exposure upon innate immunity in rodent models. Although Dean et al. (1983) reported no effects upon NK cell activity following subcutaneous injection of BaP in mice, another study (Kong et al., 1994) demonstrated that lung NK cell activity was significantly

suppressed by IT exposure of F344 rats to BaP. In addition, IT instillation of rats with BaP suppressed alveolar macrophage H_2O_2 and NO production, but not phagocytic activity (Kong et al., 1994). In contrast, *in vitro* exposure of human monocytes to BaP resulted in significantly enhanced PMA-stimulated superoxide production (Fabiani et al., 1999). Additional immune enhancing effects of BaP have also been described (Burchiel and Luster, 2001). For example, dermal BaP exposure can induce contact hypersensitivity reactions (Klemme et al., 1987; Ruby et al., 1989; Anderson et al., 1995; Ragg et al., 1995; Gorisse et al., 1999) and PAHs in diesel exhaust particles have been implicated in the induction of IgE production and type I hypersensitivity reactions (Takenaka et al., 1995).

Investigations into BaP-induced effects upon host resistance to pathogens and tumor induction using rodent models have primarily produced negative results. Contrary to the early belief that BaP could suppress tumor surveillance leading to increased tumor incidence (Stjernsward, 1969), more recent investigations have revealed that BaP exposure does not alter murine host resistance to PYB6 tumor induction (Dean et al., 1983). The more current observations regarding tumor resistance corroborate the findings that BaP exposure is only weakly associated with altered cell-mediated immunity in rodent models. Furthermore, host resistance against the bacterial pathogen *Listeria monocytogenes* was unaltered by BaP exposure (Dean et al., 1983). The pathogenesis of *L. monocytogenes* necessitates evasion of host cell-mediated immunity, whereas this immune compartment is only minimally affected by BaP exposure (Bradley, 1995). Host resistance to murine cytomegalovirus, which also

appears to rely upon evasion of cell-mediated immunity along with NK cell activity, was similarly unaltered by BaP exposure (Selgrade et al., 1988). Thus, studies employing host resistance models that rely upon evading humoral immunity are needed to more properly assess the effects of BaP-induced immunotoxicity upon susceptibility to pathogenic infection.

The determination of BaP-induced effects upon human immunity following environmental exposure presents an insurmountable task. BaP is often present in complex chemical mixtures consisting of many different PAH molecules, as well as other xenobiotics. For example, wood smoke has been demonstrated to contain various PAHs such as naphthalenes, phenanthrenes, anthracene, fluorene, fluoranthene, retene, pyrene, benzo[b,j,k]fluorene, benz[a]anthracene, and benzopyrenes (Schoket et al., 1999). In addition, tobacco smoke contains thousands of chemicals including benzo[a]pyrene. Xenobiotics present in BaP-containing environmental samples are likely to vary considerably in their individual immunotoxicities, making the determination of BaP's contribution to immune alterations very difficult. However, suppressed humoral immunity has been associated with human populations chronically exposed to BaP-containing substances such as cigarette smokers and coke-oven workers (Szczeklik et al., 1994; Moszczynski et al., 2001).

Immune dysfunction has also been reported in feral fish populations exposed to aquatic environments highly-contaminated with PAHs. Fish obtained from the Elizabeth River, a site highly-contaminated with creosote (5.6 μg BaP /g sediment), demonstrated altered immune functions compared to fish from more pristine sites

(Faisal et al., 1991). Fish sampled from contaminated Elizabeth River sites possessed significantly suppressed NCC activity, macrophage chemotaxis, phagocytosis, and respiratory burst, and lymphocyte proliferation in response to Con A (Weeks and Warinner, 1984; 1986; Weeks et al., 1987; 1990; Faisal et al., 1991). In addition, maintenance of fish in clean water for several weeks reversed the immunosuppressive effects observed in Elizabeth River fish. Interestingly, lymphocytes obtained from spot (*Leiostomus xanthurus*) recovered from contaminated sites demonstrated significantly higher responses to the B-cell mitogen LPS compared to those from clean water sites (Faisal et al., 1991). Additional evidence for immunosuppression in fish from PAH-contaminated environments is demonstrated by investigations of juvenile chinook salmon (*Oncorhynchus tshawytscha*) in the PAH- and HAH-contaminated Duwamish Waterway near Seattle, WA. Salmon from this estuary exhibited significantly reduced (compared to non-urban sites) secondary antibody production and host resistance to the bacterial pathogen *Vibrio anguillarum* (Arkoosh et al., 1991; 1998).

Several studies have demonstrated immunotoxicity following exposure of laboratory-reared fish to PAH-contaminated environmental samples. Rainbow trout (*O. mykiss*) exposed in mesocosms to liquid creosote exhibited reduced macrophage oxidative burst activity and numbers of peripheral blood B-lymphocytes (Karrow et al., 1999). Injection of chinook salmon with PAH-contaminated sediment extract (10 µg BaP /kg of sediment) resulted in significantly increased susceptibility to *V. anguillarum* challenge (Arkoosh et al., 2001). Furthermore, injection of salmon with a laboratory-prepared mixture of PAHs (6.3 µg total PAH /g BW containing 0.378 µg

BaP /g BW) significantly suppressed host resistance to the same bacterial pathogen (Arkoosh et al., 2001). Winter flounder (*Pleuronectes americanus*) exposed to crude oil-contaminated sediment for 4 months possessed significantly lower hepatic macrophage aggregates compared to flounder raised on clean sediment (Payne and Fancey, 1989).

A very limited number of studies have investigated the effects of direct exposure to BaP upon the immune response of fish. Holladay et al. (1998) demonstrated reduced splenic and pronephric lymphocyte counts and phagocyte respiratory burst activity in tilapia (*Oreochromis niloticus*) following IP injection of BaP. Altered phagocyte activity has also been reported following BaP exposure of rainbow trout (Walczak et al., 1987) and sea bass (*Dicentrarchus labrax*; Lemaire-Gony et al., 1995). Recent observations by Smith et al. (1999) reported that BaP injection could significantly reduce the PFC response of tilapia. However, BaP-induced alterations were found to be highly dependent upon the dosing schedule utilized. Faisal and Huggett (1993) exposed spot lymphocytes to BaP and the BaP metabolite BD *in vitro* and observed significant suppression of Con A-stimulated proliferation at doses as low as 1.0 nM for both compounds. Suppression of spot T-lymphocyte mitogenesis by BaP and BD was reversed by incubation of cells with ANF, suggesting a role for BaP metabolism in the observed immunotoxicity.

III. RATIONALE & EXPERIMENTAL DESIGN

A. Specific Aim 1

1. Rationale

Studies in rodent models have demonstrated that BaP exposure alters immune function (reviewed by White et al., 1994). Although suppression of the T-lymphocyte-dependent antibody response (as measured by the PFC assay) appears to be a hallmark of BaP exposure in rodents, altered innate and cell-mediated immune functions have also been described. Furthermore, rodent studies have indicated that BaP-induced immunosuppression requires metabolism of the parent compound into immunotoxic metabolites.

Limited laboratory studies in fish have demonstrated immunomodulation by BaP (Walczak et al., 1987; Faisal and Huggett, 1993; Lemaire-Gony et al., 1995; Holladay et al., 1998; Smith et al., 1999). Feral fish species inhabiting PAH-contaminated environments with measurable levels of BaP in the sediment exhibit altered immune functions compared to fish captured in more pristine sites (Weeks and Warinner, 1984; 1986; Weeks et al., 1987; 1990; Arkoosh et al., 1991; 1998; Faisal et al., 1991). Furthermore, exposure of fish (including medaka) to BaP has been demonstrated to induce CYP1A-mediated metabolism of BaP (Stegeman and Hahn, 1994).

Since fish and mammals possess morphologically /functionally similar immune systems and BaP biotransformation pathways, it appears likely that BaP will

alter the immune response of medaka. **Thus, it was hypothesized that the immune response of medaka would be altered by a single IP injection of BaP.**

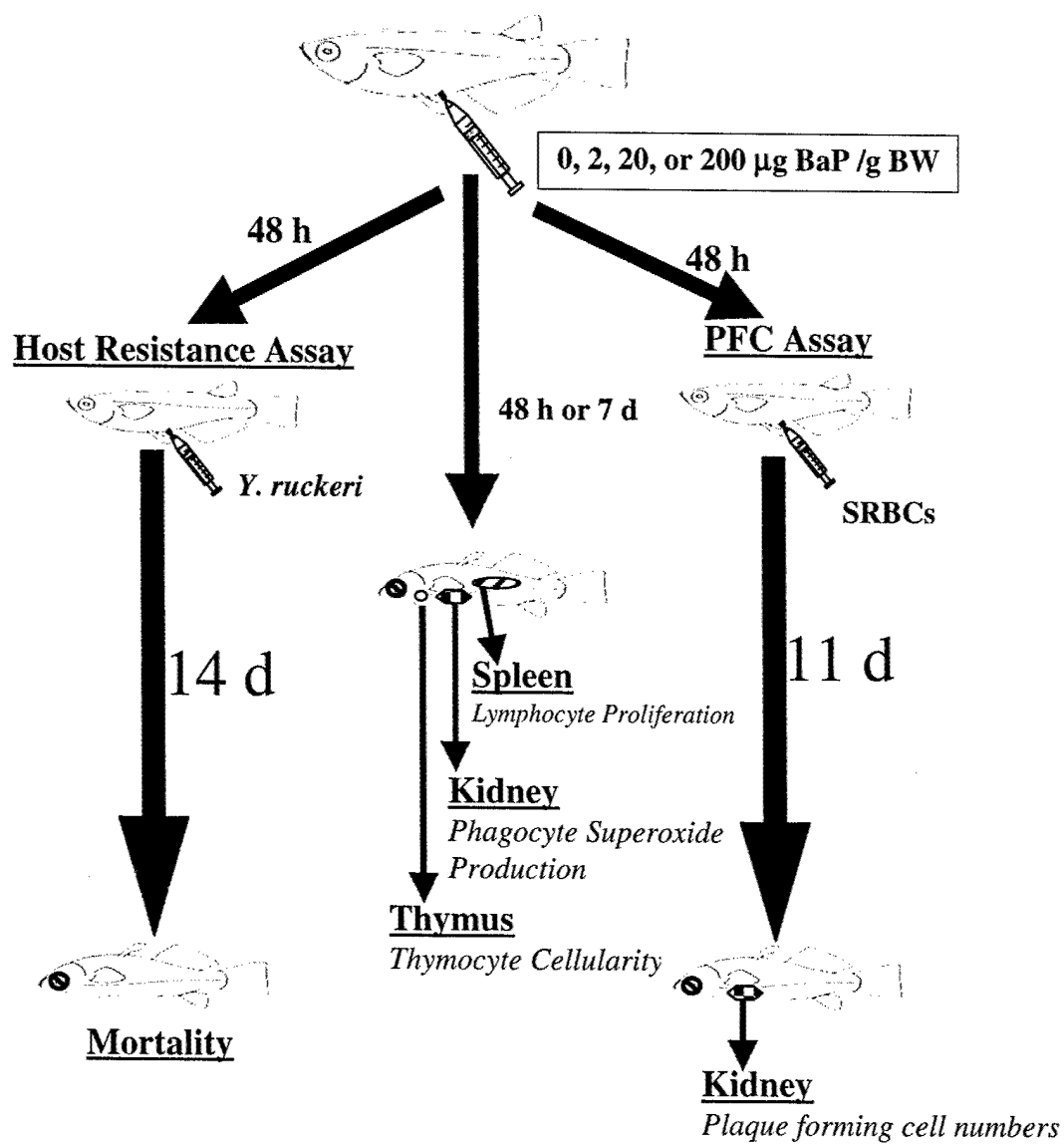
The potential of BaP to alter the immune response of medaka was investigated by employment of a battery of immune assays, originally developed in rodents (Luster et al., 1988), and adapted /validated for use in medaka. In addition, the ability of BaP to alter medaka host resistance to a bacterial pathogen was also determined. Previous and concurrent studies utilizing medaka immune functional and host resistance assays have revealed immunomodulation following laboratory exposure to cadmium, nickel, permethrin, malathion, PCB congener 126, and chemically-contaminated groundwater (Zelikoff et al., 1996; 2000; 2002; Luebke et al., 1997; Zelikoff, 1998; Beaman et al., 1999; Duffy et al., in press).

Little information exists regarding the effects of known mammalian immunotoxicants in fish species (reviewed by Zelikoff, 1994). Therefore, information gained in this study will help to further validate this fish species as an alternate model to rodents in immunotoxicological studies. In addition, information concerning BaP-induced immune alterations in medaka could be of great value for assessing the ultimate risk of environmental exposure to BaP, a pollutant commonly found in aquatic ecosystems.

2. Approach Used

Figure 2 depicts the experimental design for this Aim. In order to determine acutely non-toxic doses, medaka host survival was monitored for up to 7 d post-IP

Figure 2. Specific Aim 1 overall experimental design.



injection of BaP at either 0 (vehicle control), 2, 20, 200, 400, or 600 μg BaP /g BW. Gross external appearance and behavior were monitored throughout the exposure period and condition factors calculated at 2 and 7 d post-injection. Based upon pilot studies that indicated that the immune function of medaka was suppressed at 2 d post-BaP injection, all further investigations were conducted at this same time-point.

Effects of acute exposure to BaP upon medaka immunocompetence were examined using a battery of immune functional endpoints, as well as a bacterial host resistance challenge assay. Fish (15 – 35 fish /treatment group) received an IP injection of BaP at either 2, 20, or 200 μg /g BW or the vehicle control. Two days post-injection, fish were sacrificed and appropriate immune organs excised for determining: cellularity /viability of lymphoid organs (i.e., thymus, spleen, and kidney); lymphocyte proliferation by splenic B- and T-cells; and, kidney phagocyte-mediated superoxide production. To investigate the effects of BaP exposure upon medaka humoral immunity, SRBCs were injected 2 d post-BaP treatment and antibody-forming cell numbers enumerated 11 d later. Host resistance against bacterial challenge was also determined by infecting fish (20 – 25 fish /treatment group) with *Yersinia ruckeri* at 2 d post-BaP exposure and monitoring mortality over a subsequent 14 d period.

B. Specific Aim 2

I. Rationale

Studies in rodent models indicate that cells from lymphoid tissues are capable of metabolizing BaP into its active metabolites. Both constitutive and BaP-induced CYP1A1 expression has been demonstrated in rodent immune organs /specific cell types (Ladics et al., 1992a, b, c; Germolec et al., 1995; 1996). Furthermore, levels of both AhR mRNA and protein have been reported for rodent immune organs and leukocytes (Germolec et al., 1995; 1996; Marcus et al., 1998). Thus, it appears likely that rodent lymphoid tissues possess the capability to metabolize BaP into reactive (possibly immunotoxic) immunotoxic metabolites *in situ*.

Basal and AhR ligand-inducible CYP1A expression has also been described in certain teleost immune organs (i.e., kidney and spleen) (Stegeman and Hahn, 1994; Marionnet et al., 1997; Taysse et al., 1998). In addition, AhR mRNA has been observed in the spleen of Atlantic tomcod (Roy and Wirgin, 1997) and in the kidney of mummichog (Karchner et al., 1999). However, AhR and CYP1A expression have not been demonstrated in the immune compartments within these same organs following BaP exposure in fish (Stegeman and Hahn, 1994).

Based upon preliminary studies presented herein, it was apparent that immune cells of both the medaka kidney and spleen are adversely affected by IP BaP exposure. **Thus, it was hypothesized that IP exposure to BaP induced the expression of CYP1A in specific lymphoid organs (i.e., kidney and spleen) and immune cell**

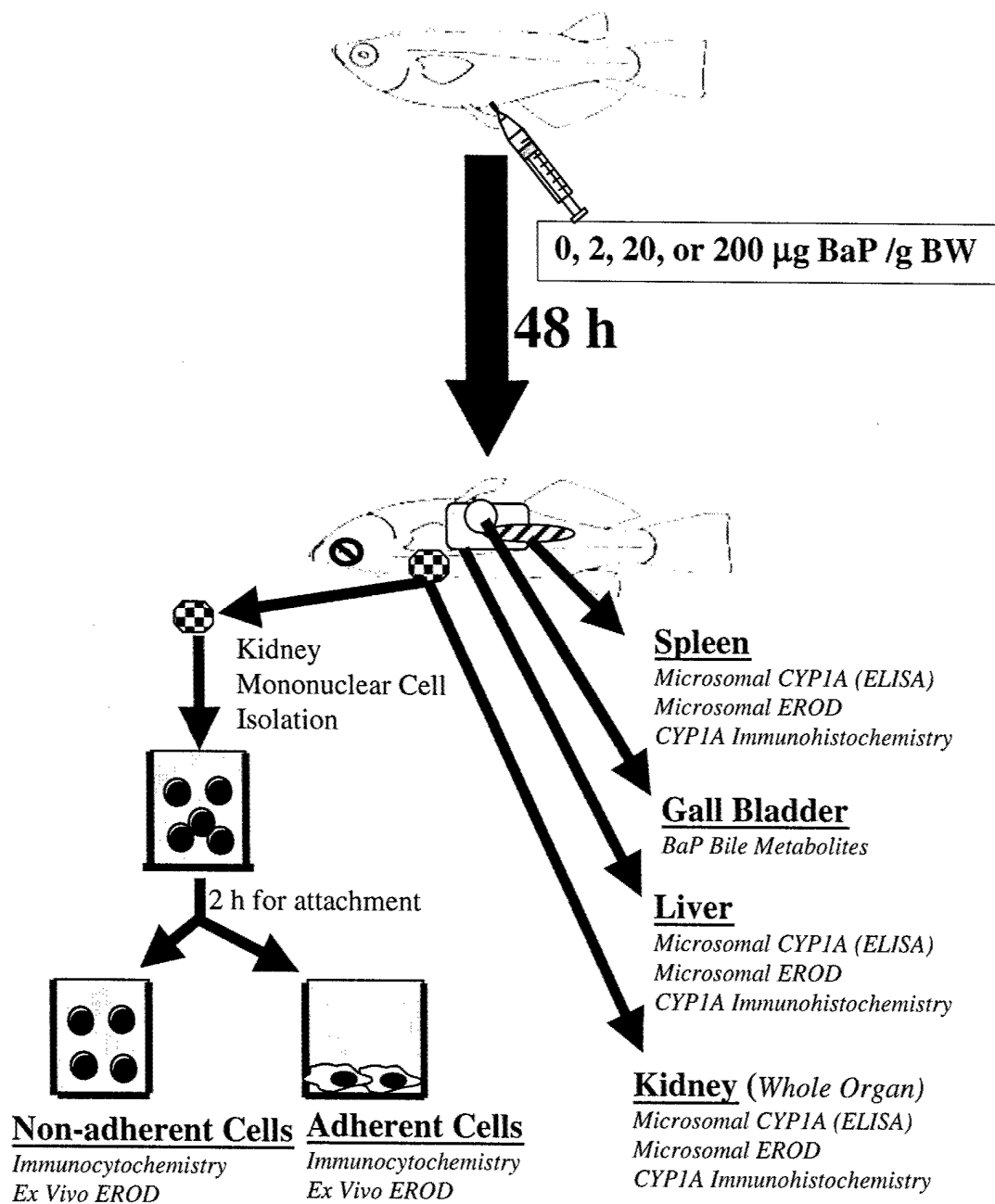
types (i.e., lymphocytes and macrophages/monocytes) of medaka. To address the aforementioned hypothesis, Specific Aim 2 investigated the potential of BaP to induce CYP1A protein and enzyme activity in medaka kidney and spleen following an exposure protocol identical to that described for Specific Aim 1. In addition, CYP1A induction was monitored in the liver (a major site of CYP1A-mediated BaP metabolism in other species).

Reactive BaP metabolites have been strongly implicated in BaP-induced immunotoxicity in mammals (White et al., 1994). CYP1A expression in specific immune organs /cells of medaka following BaP exposure demonstrates that the same potentially immunotoxic metabolites are produced *in situ* within medaka immune organs. A failure to detect CYP1A expression in immune organs /cells could indicate that: BaP-induced immunotoxicity does not require CYP1A-mediated metabolism of the parent compound (i.e., direct BaP immunotoxicity, AhR cross talk, AhR-mediated expression of genes other than CYP1A, or CYP1A-independent metabolism of BaP); BaP metabolites are transported from distant sites (Ginsberg and Atherholt, 1989; 1990); or, immunosuppression is mediated from a distant site via indirect mechanisms of immunomodulation (i.e., endocrine and hypothalamic-pituitary axis).

2. Approach Used

Figure 3 depicts the experimental design for this Aim. Effects of acute exposure to BaP upon cytochrome P450 1A (CYP1A) protein expression and enzyme activity in medaka liver and certain lymphoid organs (i.e., kidney and spleen) were

Figure 3. Specific Aim 2 overall experimental design.



determined. Two days post-IP injection of BaP at either 0 (vehicle control), 2, 20, or 200 µg /g BW, fish were sacrificed and liver, kidney, and spleen excised for determination of microsomal CYP1A protein levels and activity. Medaka gall bladders were also collected at the same post-injection timepoint and pooled for assessment of bile BaP metabolite levels.

Investigations determining the effects of BaP exposure upon medaka microsomal CYP1A expression utilized a CYP1A ELISA assay; activity of liver, kidney, and spleen microsomal CYP1A was determined by measuring EROD activity. Three to five liver microsomal samples prepared from each fish, as well as one pooled sample of either spleen or kidney (3 – 10 organs) were assessed for CYP1A protein expression per activity per treatment group per experiment.

To assess CYP1A protein expression in specific tissues and/or cell types within medaka liver, kidney, and spleen, samples from 3 – 10 fish /treatment group were analyzed for CYP1A immunohistochemistry (IHC). Mononuclear immune cells were also isolated from medaka kidney and separated into glass-adherent and non-adherent populations for analysis of CYP1A immunocytochemistry (ICC) and *ex vivo* EROD activity.

C. Specific Aim 3

1. *In Vivo* Studies

a. *Rationale*

Investigators have demonstrated that BeP, a structurally similar congener of BaP (c.f. Figure 1D), does not produce immunotoxicity in rodent models (White et al., 1994). Investigators proposed that the lack of BeP-induced immunosuppression in mammalian species may be due to the lack of reactive metabolites being formed. In addition, previous studies using rodent models have also shown that ANF can reverse BaP-induced suppression of PFC number (Kawabata and White, 1987; 1989; Ladics et al., 1991). This finding suggests that BaP metabolites may, indeed, be required for the suppression of PFC number.

However, such observations does not rule out the possible involvement of AhR-dependent events upstream to CYP1A induction, since flavone antagonists have been found to prevent AhR translocation to the nucleus following ligand binding (Henry et al., 1999). XREs are located upstream of many genes other than CYP1A1 (Nebert et al., 2000). In addition, AhR has been found to cross-talk with various other signal transduction cascades, including the estrogen, hypoxia, retinoblastoma, and NF- κ B pathways (Ge and Elferink, 1998; Caruso et al., 1999; Chan et al., 1999; Tian et al., 1999; Puga et al., 2000). Thus, compounds which do not interfere with BaP-AhR binding, yet inhibit CYP1A expression, could be of great help in gaining insight as to whether BaP metabolism is required for immunotoxicity. One such compound is DHEA, which was found to inhibit (in a post-transcriptional manner) CYP1A1 mRNA

expression and EROD activity in human cancer cells exposed to DMBA or TCDD (Ciolino and Yeh, 1999).

Since it has been hypothesized that BaP exposure of medaka results in suppression of humoral immunity (Specific Aim 1) and induced CYP1A expression /activity in medaka kidney isolated immune cells (Specific Aim 2), the inhibition of AhR signaling and/or CYP1A enzyme activity following exposure to BaP was performed so as to provide clues as to the importance of these signal transduction and biotransformation pathways in BaP-induced immunosuppression. **Thus, it was hypothesized that BaP-induced immune alteration occurred via the production of reactive BaP metabolites.** To address this hypothesis, Specific Aim 3.1 investigated the effects of exposure to either BeP or BaP in conjunction with AhR antagonists / CYP1A inhibitors ANF (c.f. Figure 1E) and dehydroepiandrosterone (DHEA; c.f. Figure 1F) upon PFC number.

By inhibiting production of BaP metabolites *in vivo*, it should be possible to determine the role(s) that BaP metabolites might play in producing immunosuppression in medaka. Also, employing compounds that do not inhibit BaP binding to the AhR but inhibit CYP1A activity may provide additional clues as to whether immunosuppression is occurring through BaP metabolism-independent mechanisms such as AhR cross-talk with other pathways, or via AhR induction of genes other than CYP1A.

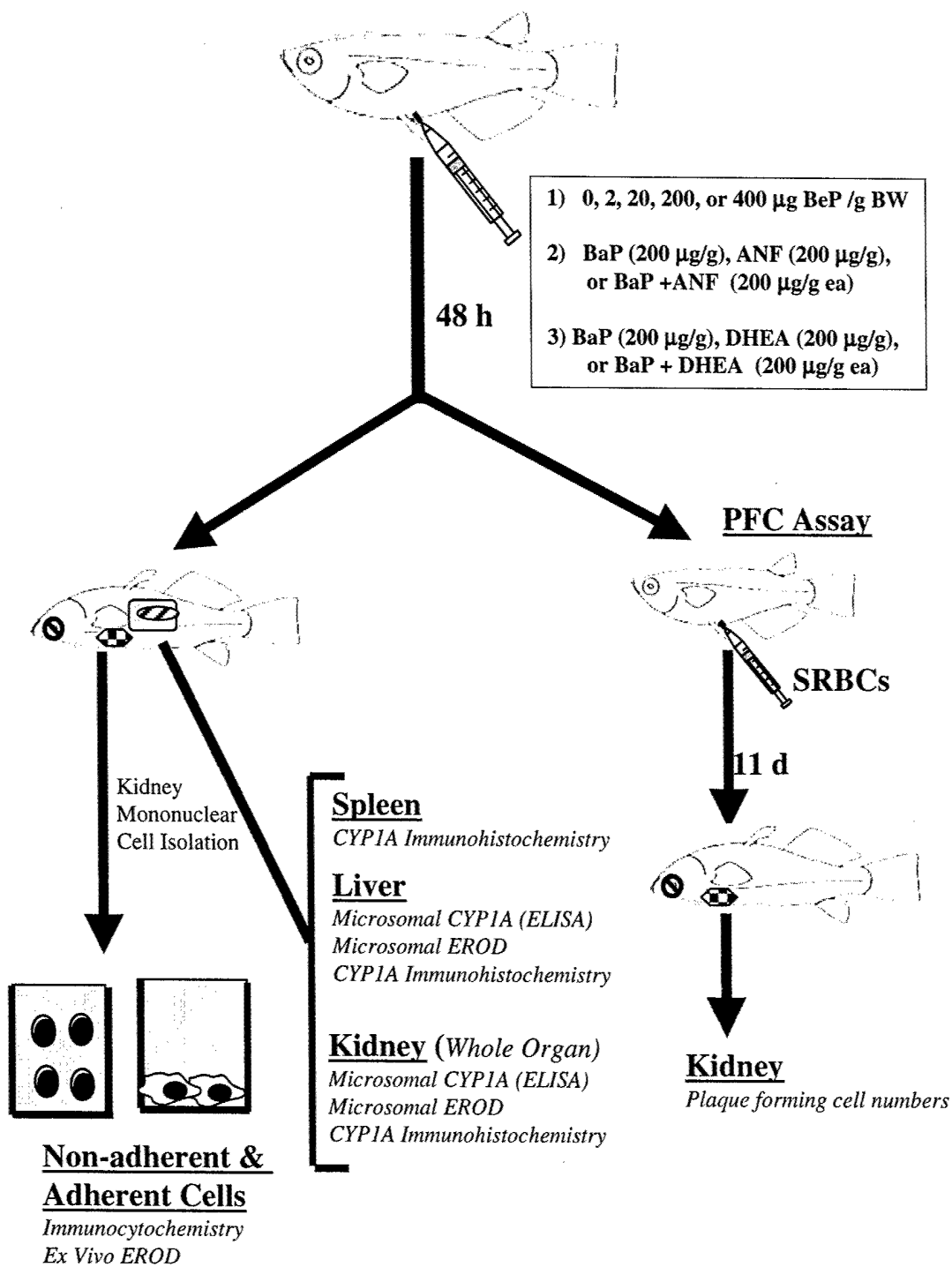
b. Approach Used

Figure 4 summarizes the overall experimental approach for Specific Aim 3.1. Fish received an IP injection of BeP at either 2, 20, 200, or 400 µg /g BW or the vehicle control. Two days post-BeP injection, medaka were immunized with SRBCs to permit later enumeration of kidney PFC numbers. In addition, microsomal CYP1A expression and activity levels were assessed for liver, kidney, and spleen by CYP1A ELISA and EROD assays, respectively; immunohistochemical localization of CYP1A within these organs was also determined.

Other studies determined the effects of either Ah receptor antagonism or CYP1A enzyme inhibition upon BaP-induced immunotoxicity by employing ANF. For these studies, fish received an IP injection of either BaP (200 µg /g BW), ANF (200 µg /g BW), BaP and ANF in combination (200 µg /g BW of each), or the vehicle control. Two days post-injection, medaka were immunized with SRBCs for later determination of PFC numbers. Expression of CYP1A following treatment with ANF was monitored in the liver, kidney, and spleen by CYP1A ELISA and IHC; CYP1A activity was assessed in the liver and immune organ microsomes using an EROD assay.

To inhibit CYP1A-mediated metabolism of BaP *in vivo* without interfering with BaP-induced expression of CYP1A (via Ah receptor activation), effects of concurrent exposure to BaP and DHEA (a steroid hormone precursor and post-transcriptional inhibitor of CYP1A activity) upon BaP-induced immunotoxicity were assessed. For these studies, fish received an IP injection of either BaP (200 µg /g BW),

Figure 4. Specific Aim 3.1 overall experimental design.



DHEA (200 µg /g BW), BaP and DHEA in combination (200 µg /g BW of each), or the vehicle control. Two days post-injection, fish were immunized with SRBCs for later determination of PFC numbers. CYP1A expression was assessed by CYP1A ELISA, IHC, and ICC; CYP1A enzyme activity was monitored by an EROD assay.

2. *In Vitro* Studies

a. Rationale

Studies investigating the effects of *in vitro* exposure of rodent cells to either BaP, BD, or BPDE have demonstrated a suppression of PFC numbers by all three compounds (Ladics et al., 1991; Kawabata and White, 1987; 1989). Additionally, ANF exposure *in vitro* abolished the observed suppression of rodent PFC number following BaP and BD exposures. Thus, rodent immune cells appear capable of directly metabolizing both BaP and BD into immunotoxic metabolites.

In the cited rodent studies, it was not determined whether the doses of ANF used inhibited events upstream of CYP1A-mediated metabolism of BaP, such as AhR cross-talk, AhR activation, or CYP1A induction. *In vitro* studies using mammalian cell cultures have demonstrated both concentration- and cell type-dependent AhR antagonist and agonist properties for ANF (Wilhelmsson et al., 1994; Henry et al., 1999). High concentrations of ANF also competitively inhibit CYP1A1 enzyme activity (Kiyohara and Hirohata, 1993). In addition, previous rodent studies investigating BaP immunotoxicity did not attempt to employ compounds that inhibit CYP1A1 activity without interfering with AhR signaling (i.e., DHEA [post-

transcriptional inhibitor of CYP1A expression], ELP [CYP1A1 suicide inhibitor; c.f. Figure 1G], or pure AhR antagonists such as 3'-methoxy-4'-nitroflavone [MNF; c.f. Figure 1H]).

Since BaP-induced immunotoxicity might be dependent upon CYP1A-mediated metabolism of BaP (Specific Aim 3.1) and medaka immune cells are hypothesized to express CYP1A following BaP exposure, it is likely that BaP and/or its metabolites may alter the function of medaka immune cells. **Thus, the hypotheses of Specific Aim 3.2 was that BaP, BD, and BPDE could directly suppress the antibody-producing capability of isolated kidney mononuclear cells. Moreover, it was hypothesized that antagonism of the AhR pathway and/or inhibition of CYP1A activity within isolated immune cells during BaP exposure would ameliorate BaP- and BD-, but not BPDE-induced immunosuppression.** To address these hypotheses, isolated kidney mononuclear cells were exposed *in vitro* to BaP, BD, or BPDE to observe whether (an the extent to which) these compounds affect PFC numbers. In addition, cells were co-incubated with BaP /BaP metabolite and AhR antagonists /CYP1A inhibitors (i.e., MNF, ELP, DHEA, or ANF separately) prior to evaluation of PFC numbers.

A demonstration of immunosuppression following *in vitro* exposure of medaka immune cells to BaP and BaP metabolites would indicate that mechanisms other than indirect modulation of the immune response occur. In addition, the prevention of BaP- or BaP metabolite-induced immunosuppression following inhibition of the AhR pathway would indicate involvement of AhR signaling and/or CYP1A-mediated

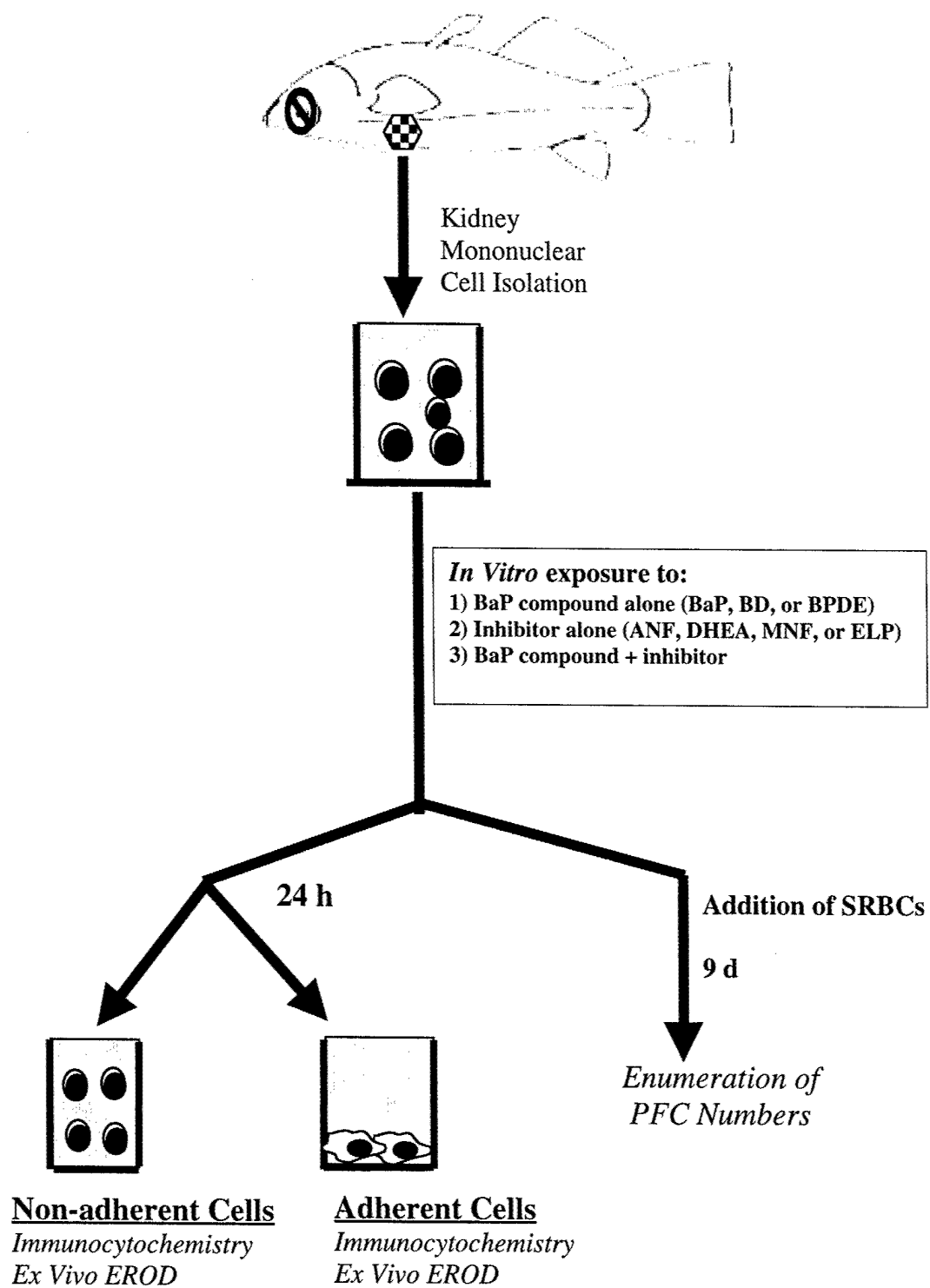
metabolism in the observed immunotoxicity. Finally, the prevention of BaP- or BaP metabolite-induced immunosuppression following inhibition of CYP1A activity, but not AhR signaling, would indicate a requirement for CYP1A-mediated metabolism for the observed immunosuppression.

b. Approach Used

Figure 5 summarizes the overall experimental approach for Specific Aim 3.2. To investigate whether direct (*in vitro*) exposure to BaP affected medaka immune cell function, isolated kidney mononuclear cells were exposed to BaP at either 0.0001, 0.01, or 1.0 μM or the vehicle control concurrent with an immunizing dose of SRBC. The number of PFCs were then assessed 9 d later. In a separate *in vitro* study, CYP1A protein expression and activity in adherent and non-adherent immune cell populations at 24 h post-BaP exposure were monitored by CYP1A ICC and *in vitro* EROD assays, respectively. In analogous studies to determine whether *in vitro* exposure of medaka immune cells to specific BaP metabolites (BD or BPDE) could alter immune functional activity, isolated kidney mononuclear cells were exposed to BaP metabolites (BD or BPDE) at either 0.0001, 0.01, or 1.0 μM or the vehicle control.

Effects of various antagonists of the AhR and/or inhibitors of CYP1A activity upon medaka immune cell function were also determined. Isolated kidney mononuclear cells were exposed to 0.05 μM of the AhR antagonist MNF in conjunction with 1.0 μM BaP, BD, or BPDE prior to enumerating PFC numbers and assessing CYP1A expression /activity. Effects upon immune function and CYP1A

Figure 5. Specific Aim 3.2 overall experimental design.



expression /activity were also determined following co-exposure to BaP or individual BaP metabolites (1.0 μ M BaP, BD, or BPDE) and 1.0 μ M of the AhR antagonist and CYP1A inhibitor, ANF. Additional studies were also performed to investigate whether compounds that do not interfere with AhR-dependent induction of CYP1A protein expression by BaP, but inhibit CYP1A-mediated metabolism of BaP, could alleviate BaP-induced immunosuppression. For these studies, isolated kidney mononuclear cells were exposed to either 1.0 μ M DHEA (post-transcriptional CYP1A inhibitor) or 0.0001 μ M ELP (suicide inhibitor of CYP1A) in combination with 1.0 μ M of either BaP, BD, or BPDE prior to enumerating PFC numbers and assessing CYP1A expression/activity.

IV. Methodology

A. Reagents and Equipment

All reagents and equipment used in this study were purchased from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

B. Animal Maintenance

Adult (8- to 10-month-old) Japanese medaka (*Oryzias latipes*, orange strain) served as the test organism for all *in vivo* studies and as the source of primary cell cultures for all *in vitro* experiments. Fish were obtained from the US Army Center for Environmental Health Research (USACEHR, Ft. Detrick, MD) and maintained at

25°C in 42 L aquaria under static-renewal conditions. All animals were allowed to acclimate for at least 7 d prior to use. A photoperiod of 16 h light: 8 h dark was maintained in the fish facility for all studies. Water quality was monitored for pH, dissolved oxygen, chlorine, temperature, and unionized ammonia at least twice a week using freshwater aquaculture colorimetric kits (LaMotte, Chestertown, MD) and the following parameters maintained: water temperature of 24 – 26 °C; pH = 6.0 – 7.0; < 0.1 mg /L unionized ammonia; > 5 mg /L dissolved oxygen; and, < 0.05 mg /L chlorine. Tap water from the Nelson Institute of Environmental Medicine served as the tank water source for all studies. Due to the relatively high chlorine concentrations in tap water, all tank water was treated overnight with 150 ml /42 L aquarium of 0.005 M sodium thiosulfate in order to remove free chlorine; chlorine levels were tested just prior to addition of fish. Medaka were fed once daily a diet consisting of Tetramin® Richmix flake food (Tetra Sales, Blacksberg, VA) and checked visually for any signs of disease; fish displaying overt signs of illness were excluded from all studies. Fish density did not exceed 35 fish / 42 L aquarium. Strict aquaculture protocols (i.e., reduction of noise and disinfection of shoes, floors, nets, and tanks) were utilized to minimize the introduction of stress into the environment.

C. Injection Protocol

Xenobiotics administered via IP injection were first dissolved in autoclaved corn oil. Chemicals utilized for *in vivo* exposures included benzo[a]pyrene (BaP), benzo[e]pyrene (BeP), α -naphthoflavone (ANF), and dehydroepiandrosterone

(DHEA). Stock solutions, consisting of either 13.3 or 6.7 mg of chemical /ml corn oil, were heated in the dark overnight in a 60° C waterbath to permit complete dissolution. Stocks were then diluted in corn oil to desired working concentrations (i.e., 13.3, 6.7, 0.7, 0.07 mg/ml for doses of 400, 200, 20, and 2 µg /g body weight (BW), respectively); corn oil alone served as vehicle control. The volume of working solution provided was based upon body weight; for example, a 0.5 g fish received an injection of 15 µl of working solution or vehicle (i.e., body weight (g) x 30 = µl of working solution). When fish were exposed to chemicals in combination, working solutions were created with the appropriate quantities of both chemicals (e.g., 6.7 mg BaP/ml and 6.7 mg ANF /ml served as the 200 µg /g BW combined dose of BaP and ANF).

Prior to injection, fish were randomly placed into 4 L plastic beakers filled with aerated tank water. Individual fish were netted, carefully blotted dry with a paper towel, their approximate wet-weight (g) determined, and the volume working solution to be injected calculated. The appropriate volume was pipetted into a 0.5 ml plastic centrifuge tube and then carefully drawn into a 28 gauge 0.5 ml plastic syringe (Beckton Dickinson, Franklin Lakes, NJ). Fish were injected IP between the paired fins located on the ventral surface, just anterior ($\approx$ 0.5 cm) to the anus. Injected fish were then gently placed into pre-determined 4 L plastic beakers containing aerated tank water. Following injection of all fish within a single exposure group, fish were transferred to a 42 L glass aquarium. Medaka were then monitored for 2 h; any mortality observed in this timeframe was deemed attributable to injection stress.

Injection protocols for the PFC and host resistance assays are described in detail in the appropriate sections (c.f. sections IV.I.3 and IV.J).

D. Assessment of Mortality, Condition Factors, Gross External Appearance, and Behavior

Exposure-induced mortality was determined for up to 7 d post-injection. Dead fish were immediately removed from the tanks to prevent growth of fungi. Mortality for each treatment group was expressed as the cumulative percent mortality by 7 d post-injection.

Condition factor (condition factor (K) = weight (g) / [length (mm)]³; Piper et al., 1992), a measure of overall fish health, was determined prior to sacrifice at 2 and 7 d post-injection. Fish were observed for any signs of external lesions or abnormalities such as: abnormal pigmentation; excessive mucus; exophthalmia (bulging of eyes); body curvature (i.e., scoliosis or lordosis); gill malformations; hemorrhage; or, skin erosions, ulcerations, or masses. In addition, fish were also monitored for any changes in behavior, including: loss of equilibrium; quiescence or hyperexcitation; surfacing or sounding; twitching or tetanus; erratic swimming; skittering or lying on their lateral surface; or, changes in respiration patterns (Brusick and Young, 1981).

E. Fish Sacrifice and Organ Dissection

At the end of the designated exposure period, fish were sterilized by swabbing with 70% (v/v) ethanol and then sacrificed by decapitation. The use of an anesthetic

(i.e., tricaine methane sulfonate [MS-222]) for euthanizing animals was avoided due to known interactions of MS-222 and other commonly-used fish anesthetics with xenobiotic biotransformation. By cutting posterior to the opercula and anterior to the pectoral fins, fish were decapitated using sterile stainless-steel surgical scissors. Since considerable interference was observed in splenic lymphocyte proliferation and kidney phagocyte superoxide assays due to the presence of erythrocytes, fish were bled prior to sacrifice by severing the caudal artery.

Following sacrifice, medaka were examined for any gross internal abnormalities such as hemorrhage or abnormal organ color. The specific organ(s) dissected were dependent upon the assay to be performed. In all cases, organs were aseptically removed with ethanol-sterilized surgical forceps and placed into appropriate buffer solutions. In some instances (i.e., bile metabolite analyses), organs were immediately flash-frozen in liquid nitrogen and stored at -70°C until used. Finally, tissues to be histologically processed (either whole fish or dissected organs) were placed in 10% phosphate-buffered formalin.

F. Processing of Tissue and Whole Organisms for Histological Analysis

Formalin-fixed samples were trimmed, placed in histological tissue cassettes, processed by placement into a graded ethanol series, cleared with xylene, and infiltrated with paraffin wax. After processing, tissue was placed in molds, and embedded in blocks of paraffin, sectioned (using a microtome) at a thickness of $5\text{ }\mu\text{m}$, and then placed upon Fisherfinest premium microscope slides or Colorfrost® /Plus

microscope slides (Fisher) for routine hematoxylin and eosin (H&E) staining or immunohistochemical analysis, respectively.

For routine histopathological analyses, slides were stained with H&E as described by Luna (1969), mounted with Cytoseal™60, coverslipped, and allowed to dry for at least 18 h. Stained tissues were then examined for any histopathological lesions, with particular emphasis placed upon examining the liver and lymphoid tissues (i.e., spleen, kidney, and thymus). The occurrence of any exposure-related effects was determined by examining: alterations in circulation (i.e., hyperemia, edema, hemorrhage); inflammatory response (i.e., granuloma, granulation tissue, abscess, necrosis, ulceration); metabolic and physical disorders (degeneration, mineralization, hemosiderosis, inclusion bodies, lipid storage, parasitic cysts); and, disturbances of growth (i.e., hyperplasia, hypertrophy, neoplasia, aplasia, hypoplasia, malformations, atrophy, metaplasia, and foci of cellular alteration).

G. Immune Cell Preparation and Determination of Immune Organ Cell

Density / Viability

Medaka kidneys or spleen were removed aseptically and placed in 30 x 15 mm tissue culture dishes containing 2 – 3 ml of sterile, ice-cold (4° C) fish physiological saline supplemented with 1% (w/v) glucose (FPS⁺; Zelikoff et al., 1996). Organs were pooled within a single treatment group and single cell suspensions recovered from organs by disruption with glass/glass tissue homogenizers. Homogenate was passed through a sterile 3 ml syringe loosely-packed with glass wool to remove RBCs and

cell/tissue debris. Cell suspensions were then placed into 15 ml centrifuge tubes, centrifuged for 10 min (at 4° C) at 400 x g, and the cell pellet resuspended in appropriate media for the designated assay. Cell numbers and viability were determined by hemacytometer counting and trypan blue exclusion, respectively. Cell yields were reported as the mean number of viable immune cells isolated per fish; cell viabilities were expressed as the percentage of viable cells recovered. Immune cell numbers were adjusted by dilution in media to achieve the desired cell concentration for the particular assay being performed. The isolation of kidney mononuclear cells by density gradient centrifugation is described in a later section (c.f. section IV.P).

H. Determination of Thymic Cellularity

Since the medaka thymus was: (1) too small to be seen macroscopically; (2) observed histologically to be intertwined with surrounding connective tissue; and, (3) physically impossible to isolate, thymic cellularity (cells / mm²) within this complex immune organ was estimated by computer-assisted microscopic image analysis of histological sections. Briefly, medaka heads, fixed in 10% formalin, were sectioned (at a 5 µm thickness), and, then stained with H&E. Thymic tissue was identified microscopically and the area determined using an Olympus BH-2 light microscope, a MTI series 68 video camera (DAGE-MTI, Inc., Michigan City, IN), and the NIH version 1.62 computer program. Thymocytes per unit area were determined from at least two sections per fish (3 fish per treatment).

I. In Vivo Immune Assays

1. Kidney Phagocyte Intracellular Superoxide ($\bullet O_2^-$) Production

The production of $\bullet O_2^-$ by phorbol myristate acetate (PMA)-stimulated and unstimulated medaka kidney phagocytes was determined via a nitroblue tetrazolium (NBT) assay (Zelikoff et al., 1996). Briefly, kidney cells were suspended in L-15 media supplemented with 5% fetal bovine serum (FBS; Gibco, Grand Island, NY), 1% penicillin / streptomycin, 0.5% L-glutamine), and 15mM HEPES buffer. Cells were placed in 96-well microtitre plates at a concentration of 6×10^5 cells /well and allowed to attach for 90 min at 30° C (pre-determined as the peak attachment timepoint). Following attachment, supernatant in each well was gently aspirated and saved for enumeration of unattached cells. The remaining cells were then incubated (at 30° C) in a 1 mg/ml NBT solution in Hank's balanced salt solution (HBSS; Gibco) for 60 min (pre-determined as the peak PMA-stimulated $\bullet O_2^-$ production timepoint). Kidney cells from some wells were stimulated with 1.0 μ g /ml PMA. In addition, 30 μ g /ml superoxide dismutase (SOD) was added to some wells to more accurately determine the specific amount of $\bullet O_2^-$ produced. At least 3 replicate wells per each reaction mixture (i.e., unstimulated with SOD, unstimulated without SOD, stimulated with SOD, and stimulated without SOD) were included for each assay.

Production of insoluble formazan from NBT was monitored spectrophotometrically at a wavelength of 620 nm following cell lysis with 120 μ l 2 M KOH and solubilization with 140 μ l dimethyl sulfoxide (DMSO). Since oxidants other than $\bullet O_2^-$ (i.e., H_2O_2) can reduce NBT, the amount of formazan produced specifically

by $\bullet\text{O}_2^-$ was determined by subtracting the mean optical density (O.D.) of wells with SOD from that of wells without SOD; stimulated $\bullet\text{O}_2^-$ production was determined from wells receiving all of the aforementioned reagents plus PMA. Total nmoles $\bullet\text{O}_2^-$ were determined by multiplying the final mean O.D. values by a constant 15.87 (Pick and Mizel, 1981); data for both PMA-stimulated and unstimulated $\bullet\text{O}_2^-$ production were expressed as nmoles of $\bullet\text{O}_2^-$ produced / 6×10^5 cells / 60 min.

2. *Splenic Lymphocyte Proliferation*

The proliferation of medaka splenic lymphocytes was measured colorimetrically in a 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) reduction microtitre assay (Beaman et al., 1999). MTT is reduced by the succinate-tetrazolium reductase system of the mitochondrial respiratory chain, and production of water-soluble formazan dye has been found to correlate with the number of metabolically-active cells present. Splenocytes were suspended in L-15 media supplemented with 5% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 0.2% gentamycin, 1% penicillin / streptomycin, and 15 mM HEPES buffer and plated into 96-well microtitre plates at a concentration of 5×10^5 cells /well. Lymphocyte proliferation was determined in response to concanavalin A (Con A; 100 $\mu\text{g}/\text{ml}$) or lipopolysaccharide (LPS; 200 $\mu\text{g}/\text{ml}$) for T- and B-lymphocytes, respectively. At least three wells per reaction mixture (i.e., no mitogen, with Con A, or with LPS) were included for each assay.

Following a 3 d incubation at 30° C, 20 µl of MTT (5 mg/ml) was added to each well. The reaction was halted after 4 h by addition of 10 µl of 10% sodium dodecyl sulfate /0.01 N HCl solution. The soluble formazan product of MTT reduction was measured spectrophotometrically at 600 nm. Proliferation was calculated as the mean change in absorbance between the mitogen-stimulated and unstimulated cells.

Since MTT reduction is only an estimation of the number of proliferating cells present in cultures, and unlike ³H-thymidine incorporation does not directly measure DNA replication, the number /viability of splenocytes per culture were determined following a 3 d incubation with mitogens in order to determine whether exposure-related changes in MTT reduction correlated with cell density/viability of each culture. In this case, cell culture supernatants were removed from each well following mitogen exposure and individual wells washed 5X with HBSS. Non-adherent cells were pelleted by centrifugation (400 x g for 10 min at 4° C) and resuspended in HBSS prior to determination of cell number and viability via hemacytometer counting and trypan blue exclusion, respectively. Mean cell numbers/ culture and viability were determined for the Con A, LPS, and no mitogen treatment groups.

3. *Plaque Forming Cell (PFC) Assay*

Medaka T-lymphocyte-dependent antibody production following *in vivo* immunization with SRBCs was assessed by the method of Jerne and Nordin (1963) modified for use in medaka by Beaman et al. (1999). Two days post-chemical injection, medaka were immunized IP with 15 µl of a 10% suspension (v/v) of SRBCs

(Lampire Biological, Pipersville, PA) in Dulbecco's phosphate buffered saline (DPBS; Gibco). SRBC-injected fish were returned to their tanks and monitored for injection stress-related mortality for 2 h. Eleven days post-SRBC immunization (pre-determined time-point for optimal PFC numbers), medaka were sacrificed, and their kidney cells isolated and suspended at 2×10^7 cells / ml in L-15 media. Cell suspensions (100 μ l) were combined in 12 x 75 mm polypropylene tubes with 100 μ l of a 1.5% (w/v) solution of warm (45° C) agarose, 100 μ l of 2X L-15 media, and 200 μ l of a 10% (v/v) suspension of SRBCs. The resulting mixture was vortexed for 3 sec, poured immediately onto glass microscope slides, and incubated (at 30° C) for 4 h in a humidified chamber. Following incubation, 900 μ l of diluted guinea pig complement (diluted 1:15 with DPBS) was layered over the entire SRBC lawn. Slides were then incubated overnight at 30° C and the numbers of plaques per slide (5 slides / treatment group) enumerated after 18 – 24 h. Data were expressed as the mean plaque number per 2×10^6 kidney cells.

J. Host Resistance Challenge Assay

Forty-eight hours post-BaP injection, fish (20-30 fish per treatment group) were IP-administered an LD₁₅₋₃₀ dose of *Yersinia ruckeri* (CDC strain 2396-61; American Type Culture Collection, Manassas, VA) in 20 μ l of HBSS. The final LD₃₀ bacterial dose (2×10^6 colony-forming units [CFUs] per fish as pre-determined from pilot studies with naive fish) was calculated one day prior to injection from thawed bacterial stocks diluted in HBSS, plated on tryptic soy agar (TSA) plates, and

incubated 24 h at 37 °C. To confirm the exact number of bacteria injected, serial dilutions of the bacteria suspension used for injections were plated on agar plates and enumerated the following day. Host survival was monitored from 2 h up to 14 d and percent cumulative mortality calculated at 3, 7, and 14 d post-bacterial challenge.

K. Determination of Bile BaP Metabolites

The relative amount of BaP metabolites present in medaka bile following IP injection to BaP was estimated by fixed-wavelength fluorescence (FF) using a modification of the method of Lin et al. (1996). Gall bladders were pooled within treatment groups (5 – 10 organs /group), placed in 1.5 ml microcentrifuge tubes, and flash-frozen in liquid nitrogen prior to storage at -70° C. For analysis, samples were thawed completely before addition of 0.1 ml double distilled-water (ddH₂O), grinded with a teflon pestle, and centrifuged at 4° C for 20 min at 12,000 x g. Supernatants containing diluted bile and any water-soluble metabolites were carefully aspirated and total protein concentrations determined for each sample.

Diluted bile samples were adjusted to a protein concentration of 5 µg /ml with ddH₂O and plated in triplicate at 200 µl /well in a 48-well microtitre plate. The fluorescence of each sample was measured at excitation and emission wavelengths of 380 nm and 430 nm, respectively (peak absorbance for five-ring aromatic compounds). Data was expressed as mean relative FF /µg protein for each treatment group.

L. Tissue Microsome Preparation

Microsomal samples were prepared from individual fish livers and pooled spleen and/or kidney tissue (3 – 10 organs per microsomal sample) using the method of Nilsen et al. (1998). Originally, tissues were immediately flash-frozen in liquid nitrogen prior to microsome preparation. Further studies using microsome preparations created directly following fish sacrifice and prior to freezing proved that the original method resulted in significant reduction of microsomal enzyme activity (c.f. section V.B.3). Thus, all latter studies utilized microsomal samples prepared from fresh medaka tissue.

Briefly, tissue was placed in 1 ml microsomal homogenization buffer (MHB; pH 7.4, 0.1M sodium phosphate, 0.015M KCl, 1mM EDTA, and 1mM DTT) in a glass /glass tissue homogenizer. Tissue, completely disrupted by 20 strokes each of the tight- and loose-fitting pestles, was then transferred into 1.5 polystyrene microcentrifuge tubes. Samples were centrifuged for 20 min at 12,000 x g (4° C) and supernatants containing the post-mitochondrial fraction were collected and placed in pre-chilled (-20° C) ultracentrifuge tubes. Tubes were balanced with MHB and centrifuged for 2 h at 100,000 x g (at 4° C). The supernatant (i.e., cytosol fraction) was discarded and the microsomal pellet resuspended in 200 - 300 µl microsomal resuspension buffer (MRB; pH 7.4, 0.1 M sodium phosphate, 0.015 M KCl, 1 mM EDTA, 1 mM DTT, and 20% [v/v] glycerol). Microsomal fractions were aliquoted (10 – 20 µl / tube) into 0.5 ml microcentrifuge tubes and stored at -70° C until analyzed.

Total protein levels in the microsomal samples were determined using a BioRad DC protein assay kit (BioRad, Hercules, CA) that employed bovine serum albumin (BSA) standards.

M. Analysis of Microsomal CYP1A Protein Levels

1. Western Blotting

The estimated molecular weight and relative concentrations of medaka CYP1A protein were determined for microsomal samples originally by Western blotting. Microsomal samples (10 - 20 µg of protein / lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the method of Laemmli (1970) using 4% stacking / 10% separation gels and a Mini-Protean® electrophoresis unit (BioRad). Wide-range molecular weight markers were also included for estimation of medaka CYP1A protein molecular weight.

Following SDS-PAGE separation, electrophoresed gels were equilibrated with transfer buffer [3 g/L Tris base, 14.4 g glycine, 0.1 % (w/v) SDS, and 5 % (v/v) methanol] and gel proteins were transferred to NitroBind nitrocellulose membranes using a Mini Trans-Blot® electrophoretic transfer cell (BioRad). Following blotting, membranes were washed extensively in Tris buffered saline with Tween-20 (TBST; 20 mM Tris-HCl, 500 mM NaCl, 0.1% (v/v) Tween-20, pH 7.5). Lanes containing the molecular weight markers were removed for later analysis. Membranes were blocked with TBST containing 2.5% (w/v) nonfat dry milk (Hain Food Group, Uniondale, NY), and rewashed with TBST prior to addition of a 1:3 dilution in (TBST) of primary

antibody (i.e., the monoclonal antibody hybridoma supernatant, C10-7; gift from Dr. Charles Rice, Clemson Univ., SC) generated against a polypeptide sequence from rainbow trout (*O. mykiss*) CYP1A1 (Rice et al., 1998). Following overnight incubation at 4° C, membranes were washed extensively with TBST before incubation (90 min at 37° C) with the secondary antibody (anti-mouse IgG alkaline phosphatase conjugate; Sigma) diluted in TBST. After incubation, the membranes were washed with TBST and then with TBS without Tween-20 prior to the addition of the 5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium (BCIP/NBT) enzyme substrate. The reaction was halted by placement of the membrane in distilled water. Membrane sections containing molecular weight marker lanes were stained with Ponceau S to visualize marker bands for calculation of the molecular weights (kiloDaltons, KD) of any immunopositive proteins.

2. *Enzyme-Linked Immunosorbant Assay (ELISA)*

The amount of CYP1A protein in microsomal samples was estimated semi-quantitatively by a modification of the protocol of Nilsen et al. (1998). Microsomal samples were diluted in ice-cold coating buffer (50 mM carbonate / bicarbonate, pH 9.6) to produce concentrations of 10 µg microsomal protein /100 µl for liver and 20 µg microsomal protein /100 µl for kidney and spleen. Samples (100 µl /well) were added in either duplicate or triplicate to individual wells of an Immunolon #4 96-well microtitre plate (Dynatech Labs, Chantilly, VA) and incubated overnight at 4° C. After 18 –24 h, wells were thoroughly washed extensively with PBS (pH 7.3;

supplemented with 0.05% [v/v] Tween-20 [TPBS]) prior to an 1 h incubation (at RT) with blocking buffer (2% [w/v] BSA in PBS). Following incubation with the blocking buffer, wells were again washed with TPBS. One hundred microliters of primary antibody solution (monoclonal antibody C10-7 diluted 1:4 in 1% BSA in PBS) was then added to each well prior to overnight incubation (at 4° C). The following day, wells were washed with TPBS and then incubated for 1 h (at RT) with secondary antibody solution (anti-mouse IgG alkaline phosphatase conjugate diluted 1:1000 in 1% [w/v] BSA /PBS). Wells were washed subsequently with TPBS and then PBS prior to addition of the substrate (i.e., p-nitrophenyl phosphate [p-NPP]). The reaction was halted after 30 min by the addition of 3 N NaOH and plates read spectrophotometrically at 405 nm. The relative amount of CYP1A protein in each microsomal sample was expressed as mean O.D. units obtained by subtracting the mean absorbance of sample wells without primary antibody (negative controls) from the mean absorbance of wells with the primary antibody.

N. Microsomal EROD Assay

CYP1A enzyme activity in medaka microsomes was determined using a 7-ethoxyresorufin *O*-deethylase (EROD) assay (Hahn et al., 1993). Microsomal samples were diluted to a concentration of either 10 µg (for liver) or 20 µg (for kidney and spleen) per 30 µl of 50 mM Tris-NaCl buffer, pH 7.4 (TN). Thirty microliters of diluted microsomal samples were added in duplicate or triplicate to individual wells of a 96 well microtitre plate. Resorufin standards in TN buffer were also included (in

triplicate) on each plate at 0 - 250 pmoles / well. One hundred fifty microliters of 2.67 μ M 7-ethoxyresorufin (in TN) was added to each well prior to initiating the EROD reaction by addition of 20 μ l of 10mM β -nicotinamide adenine dinucleotide phosphate (NADPH) cofactor. Fluorescence was measured every 2 min (for a total of 20 min) with an HTS 7000 Bioassay Reader (Perkin-Elmer Inc., Norwalk, CT) using excitation and emission wavelengths of 535 and 590 nm, respectively. EROD activity was calculated as pmoles of resorufin produced /min /mg protein, using the linear portion of the kinetic curve.

O. CYP1A Immunohistochemistry (IHC)

The relative amount of CYP1A protein present within each individual cell/tissue type was assessed using a modification of the protocol of Stegeman et al. (1991). Tissue sections on Colorfrost® /Plus microscope slides were heated in a oven at 60 °C for at least 2 h prior to emersion in xylene to clear paraffin wax from the tissue. After 10 min, slides were placed in fresh xylene for an additional 10 min. Slides were then sequentially soaked twice in absolute, 90% and 70% ethanol (3 min each), prior to gentle rinsing in tap water for 30 s. Tissues were then allowed to equilibrate in 0.01M PBS (pH 7.4) for at least 1 h.

Slides were gently blotted, carefully wiped dry around the sections, and a circle drawn around each section with a wax pen. One hundred microliters of blocking buffer (10% [v/v] normal goat serum in PBS) was applied to completely cover each tissue section and slides were incubated for 1 h at RT. The slides were then rinsed with

PBS to remove blocking buffer and 100 µl primary antibody solution [1.5 µg of monoclonal antibody 1-12-3 /ml in 1% BSA in PBS; a gift from Drs. John J. Stegeman (Woods Hole Oceanographic Institute, MA) and Harry V. Gelboin (NCI)] was then added. Monoclonal antibody 1-12-3 is specific for scup (*Stenotomus chrysops*) cytochrome P450E (now confirmed to be CYP1A1) and has a very broad range of cross-reactivity with CYP1A1 from various species (Park et al., 1986). Negative control slides lacked monoclonal 1-12-3 antibody and received an equal concentration of non-specific mouse IgG in 1% BSA/PBS. Slides were then incubated overnight at 4° C with the primary antibody solution.

Following the incubation, slides were washed with PBS, blotted dry, and the sections stained (according to the manufacturer's instructions) using ExtraAvidin® alkaline phosphatase staining kit and Fast Red TR /naphthol AS-MX substrate solution. Briefly, 100 µl of secondary antibody solution (1:20 diluted biotinylated goat anti-mouse IgG in 1% BSA /PBS) was applied to each section and incubated at RT for 30 min. Slides were then rinsed with PBS and 100 µl of 1:20 diluted ExtrAvidin – alkaline phosphatase (in 1% BSA/PBS) was placed on each section. Slides were incubated for 20 min (at RT), rinsed with 0.05 M Tris-buffered saline (pH 8.0; TBS), and 100 µl Fast Red substrate solution then added. Slides were incubated 5 – 20 min; staining was terminated before generalized background staining became evident by gently rinsing in distilled water. In some instances, sections were counterstained with Mayer's hematoxylin to stain cell nuclei. All slides were then mounted with an aqueous PBS: glycerol solution (1:1) and then coverslipped.

The relative amount of CYP1A expression was evaluated for various cell/tissue types by scoring the extent of staining within the individual organs of separate fish. Staining was assessed on two levels as previously described (Guiney et al., 1997). First, by staining frequency of particular cell/tissue types was scored as follows: 0 = absence of positively-stained cells; 1 = rare staining; 2 = multifocal staining; and, 3 = many / most cells stained. Since such scoring lends no reference to the relative intensity of staining, cell/tissue types within an organ were further scored based upon staining intensity as follows: 0 = no apparent; 1 = very weak; 2 = modest; 3 = moderate; 4 = strong; and, 5 = intense. The staining frequency and intensity scores for a particular cell/tissue type within a given organ were then multiplied to obtain a "CYP1A IHC Index"; indices ranged from 0 to 15. An identical scoring protocol was used successfully in other studies to correlate IHC staining of CYP1A in liver sections ($r^2 = 0.982$) with the relative amounts of CYP1A protein (determined by Western blots) derived from the same tissue (Guiney et al., 1997). At least two sections per individual cell/tissue type within each organ were evaluated for CYP1A staining. Negative control sections (i.e., non-specific primary antibody) yielded no apparent background staining. Mean CYP1A IHC Indices were obtained for each treatment group (3 - 10 fish/group) within an experiment for the following cell/tissue types; hepatocytes, renal tubules, kidney hematopoietic tissue, and splenic endothelia.

P. Isolation of Kidney Mononuclear Cells

In order to obtain purified suspensions of kidney mononuclear cells, medaka kidneys (15 – 70 per treatment group) were suspended on ice in 3 - 5 ml of FPS⁺ supplemented with heparin (30 units/ml; Gibco). Tissues were disrupted with glass/glass tissue homogenizers in order to obtain single cell suspensions. Cell suspensions were then gently layered on top of 5 ml of Histopaque in a 15 ml centrifuge tube, and the suspensions centrifuged at 4° C for 45 min at 100 x g. Following centrifugation, yellowish-white colored bands (containing mononuclear cells) seen at the FPS⁺ / Histopaque interface were removed using a Pasteur pipette and placed in a separate 15 ml centrifuge tube containing 10 ml FPS⁺ without heparin. Isolated mononuclear cells were then pelleted by centrifugation (400 x g for 10 min at 4° C) and washed an additional two times with FPS⁺ prior to resuspension in 2-3 ml of appropriate media. Cell numbers / viability were determined by hemacytometer counting and trypan blue exclusion, respectively. Cell concentrations were adjusted by dilution with the appropriate media to the desired density (cells /ml) for the assay being performed.

Q. Kidney Mononuclear Cell Exposure

The following chemicals were used in the *in vitro* exposure system; BaP, BeP, ANF, DHEA, ellipticine (ELP), 3'-methoxy -4'-nitroflavone (MNF; a gift from Dr. Thomas A. Gasiewicz, Univ. of Rochester School of Medicine), benzo[a]pyrene -*trans* -7,8 -dihydrodiol (+/-) [BD] and benzo[a]pyrene -*r*-7,*t* -8-dihydrodiol-*t*-9,10-

epoxide (+/-)(anti) [BPDE] (both from NCI Chemical Resource Repository, Kansas City, MO). Xenobiotics used for *in vitro* experiments were dissolved completely in DMSO to prepare 1 – 2 mM stock solutions. Prior to experiments, stock solutions were diluted in DMSO to create working solutions which would result in xenobiotic concentrations ranging from 0.0001 to 1.0 μ M and a DMSO concentration of 0.5% (v/v) when added to cultures. Exposure of cells to chemicals in combination was accomplished by preparing working solutions that contained the appropriate concentration of both chemicals (i.e., 1.0 μ M BaP with 1.0 μ M ANF dissolved in DMSO). Vehicle control treatment groups received 0.5% (v/v) DMSO.

The final cell density used for all *in vitro* experiments was 5×10^6 kidney mononuclear cells /ml supplemented L-15 medium. All exposures were conducted in humidified chambers at 30° C. In addition, both adherent and non-adherent isolated kidney mononuclear cells were identified by the presence of non-specific esterase activity. Non-specific esterase activity is primarily found within cells of the monocytic lineage (i.e., monocytes and macrophages) and usually absent in lymphocytes and granulocytes. Cells were incubated in the presence of α -naphthyl acetate and a stable diazonium salt; non-specific esterase activity hydrolyzes ester linkages to release naphthol compounds that then couple with diazonium salts to produce black granular deposits within the cellular cytoplasm. One hundred adherent /non-adherent cells per treatment group were assessed for esterase-positive staining; data was expressed as the mean percentage of esterase-positive cells.

R. CYP1A Immunocytochemistry (ICC)

Cells receiving *in vivo* and *in vitro* exposures were assessed for CYP1A ICC using a modification of the protocol of Hahn et al. (1993). Kidney mononuclear cells obtained from fish exposed *in vivo* 48 h previously were utilized to determine CYP1A protein induction *ex vivo*. *In vitro* – exposed medaka kidney mononuclear cells were also assessed for CYP1A protein induction 24 h following chemical exposure. In both cases, glass-adherent and non-adherent cell populations were analyzed.

For the *ex vivo* studies, isolated kidney mononuclear cells were suspended in L-15 media (c.f. section IV.I.2) at 1×10^6 cells/ml. One milliliter of cell suspension was added to each chamber of a double-welled chamber slide, and the slides incubated for 2 h at 30° C to allow for cell attachment. Following incubation, non-adherent cells were recovered and placed in a 15 ml centrifuge tube; both chambers were washed twice with 1 ml DPBS.

In vitro exposures were conducted in double-welled chamber slides and similarly processed 24 h following treatment to obtain non-adherent and glass-adherent cell populations. Non-adherent cell suspensions were centrifuged at 4° C for 10 min at 400 x g and then resuspended in 200 – 500 µl DPBS. One hundred microliters of non-adherent cells were allowed to electrostatically adhere (for 45 min at RT) within circles pre-drawn on specially-coated Colorfrost® /Plus slides.

Supernatants from both the originally adhering and now electrostatically-adhering (i.e., originally non-adherent) cell preparations were carefully discarded and the cells fixed by the addition of 4% (w/v) paraformaldehyde (in DPBS) for 10 min.

After fixation, cells were permeablized with 0.5% (w/v) Triton X-100 (in DPBS) for 5 min prior to extensive washings with DPBS. Slides were then allowed to equilibrate with 0.01M phosphate buffered saline, (pH 7.4) for at least 1 h before being blocked for 15 min with 10% goat serum in PBS (100 µl per circle). After blocking, slides were washed with PBS and incubated at 4°C with 100 µl primary antibody solution (1.5 µg /ml of monoclonal antibody 1-12-3 in 1% BSA /PBS) for 18 – 24 h. From this point, procedures were exactly the same as those described in detail in section IV.O.

Amount of CYP1A expression was evaluated for each cell preparation by assessing staining intensity in at least 75 cells / slide. Cell staining intensity was recorded as follows: 0 = no apparent staining; 1 = weak; 2 = moderate; and, 3 = intense. The relative occurrence of CYP1A-positive cells within each treatment group was recorded as the percentage of cells staining positive for CYP1A (termed “% CYP1A Positive”). The relative frequency of cells expressing moderate- to intense-staining (i.e., a score of 2 or 3) divided by the number of positively-staining cells (termed “CYP1A ICC Index”) was calculated in order to assess changes in the magnitude of CYP1A protein expression. Published studies have shown a high correlation between a similar cell scoring protocol and CYP1A protein levels determined by Western blot within polychlorinated biphenyl (PCB)-exposed fish cell cultures (Guiney et al., 1997).

A similar protocol was also applied to non-adherent spleen cells following exposure to mitogens (c.f. section IV.I.2) in order to assess the effects of *in vitro* mitogen stimulation upon cells recorded from fish exposed to BaP *in vivo*. Briefly, cell

culture supernatants were removed (c.f. section IV.I.2) 24 h following mitogen treatment and assessed by ICC for CYP1A.

S. Ex vivo /in vitro EROD Analysis

The catalytic activity of CYP1A enzymes in viable medaka kidney mononuclear cells was estimated using a modification of the *in vitro* EROD protocol of Hahn et al. (1996). EROD activity was assessed in adherent and non-adherent kidney cells obtained by identical protocols as those described in section III.Q, except that 48-well microtitre plates were used. Briefly, following attachment (2 h for *ex vivo* studies) or 24 h post-initiation of *in vitro* exposures, non-adherent cells were removed, placed in a 15 ml centrifuge tube, and each chamber washed twice with 1 ml DPBS (RT). Phosphate buffer (PB; 50 mM Na₂HPO₄, pH adjusted to 8.0 with 50 mM NaH₂PO₄) was added to each chamber containing adherent cells (100 µl /well). Non-adherent cell suspensions were centrifuged 4° C for 10 min at 400 x g, resuspended in 100 µl PB, and then added to empty wells of the same 48-well microtitre plate. The reaction was initiated by addition of 100 µl EROD reaction mixture (4 µM 7-ethoxyresorufin, 18 µM dicumarol, and 1 mM NADPH in PB) to each well. Resorufin (0-125 pmoles / well) and BSA (0 – 100 µg /well) standards were also included (in duplicate) on each plate. The reaction was halted after 60 min by the addition of 150 µl /well of ice-cold fluorescamine solution [150 µg /ml in acetonitrile]. Plates were then incubated an additional 15 min prior to measurement of fluorescent resorufin (using 535 nm excitation /590 nm emission) and fluorescamine (using 405 nm

excitation /465 nm emission). EROD activity was calculated as pmoles resorufin formed /min /mg protein compared to the resorufin and protein standards.

T. Mishell-Dutton PFC Assay

T-lymphocyte-dependent antibody production of medaka kidney mononuclear cells was assessed *in vitro* by a modification of the protocol of Arkoosh and Kaattari (1992). Isolated kidney mononuclear cells (1×10^7 /ml in supplemented L-15 media) were added to each well of a 96-well microtitre plate (50 μ l /well). SRBCs were washed (as described in section IV.I.3) and resuspended in supplemented L-15 media at a concentration of 1×10^8 cells /ml. Fifty microliters of SRBC suspension was then mixed with the cell suspensions to yield a 1:10 kidney cell: SRBC ratio prior to the addition of 0.5 μ l of test chemical working solution or vehicle control (DMSO). All cultures were prepared in triplicate; non-immunized controls (minus SRBCs) were also prepared for each treatment group. Plates were mixed on an orbital rotator for 30 min and then placed in humidified chambers held at 30° C. Every second day, 10 μ l of supplemented L-15 media was added to each well; plates were mixed prior to re-incubation at 30° C.

Nine days after culture initiation (peak timepoint for numbers of specific antibody-producing cells /culture), covering media was removed, added to individual 15 ml centrifuge tubes, and wells containing adherent cells washed five times with 100 μ l DPBS /wash. Covering media containing non-adherent cells were then centrifuged at 400 x g for 10 min (at 4° C). Recovered cell pellets were resuspended in

supplemented L-15 media (100-200 μ l) and assessed for PFC number using an assay protocol identical to that described in section IV.I.3. A few μ l of each cell suspension was set aside for later determination of mononuclear cell number and viability by hemacytometer counting and trypan blue exclusion, respectively. Following plaque enumeration, data were expressed as the mean number of plaques / 1×10^6 cells. To obtain the number of SRBC-specific plaques, the number of plaques (per 1×10^6 cells) from cultures lacking SRBCs was subtracted from the number of plaques (per 1×10^6 cells) formed in cultures containing SRBCs.

U. Statistical analysis

Differences between exposure groups were determined using a one-way analysis of variance (ANOVA) followed, when necessary, by Tukey's post-hoc testing. Relationships between experimental protocols (i.e., different microsomal preparation techniques) were analyzed by regression analysis. All statistical calculations were performed using SuperANOVA software (Abacus Concepts, Berkeley, CA). At least 3 individual experiments were performed for each end-point (i.e., $n = 3$) and data was expressed as the mean $\pm$ standard error of the mean (S.E.M.).

V. Results

A. SPECIFIC AIM 1

1. *General Health Indices*

Intraperitoneal injection of 2 – 600 BaP $\mu\text{g/g}$ BW had no effect upon host survival for up to 7 days post-injection (Table 2). Medaka condition factors were also unaffected by BaP exposure in this same dose range for up to 7 d post-injection. At the highest dose (i.e., 600 μg BaP /g BW), it became apparent that the BaP was not completely dissolved, indicating that this BaP dose was at or near maximum solubility in corn oil. Additionally, no noticeable changes in medaka behavior or gross external appearance were observed at any dose examined. Furthermore, gross examination for internal pathologies 2 d post-BaP injection revealed no chemical-related lesions.

2. *Immune Organ Cellularity & Viability*

A single IP injection of BaP at a dose as high as 200 $\mu\text{g/g}$ BW had no effect upon kidney and spleen cell yields or viabilities at 2 d post-injection (Table 3). Moreover, computer-assisted video microscopy revealed that cellularity of the thymus gland was also unaltered by BaP treatment 2 and 7 d post-injection. No significant associations were discerned between BaP exposure and the presence of histopathological lesions in medaka kidney or spleen.

Table 2.

Effects of benzo[a]pyrene (BaP) upon general health indices of Japanese medaka 48 h and 7 days post-injection^a

BaP concentration ($\mu\text{g BaP /g BW}$)	Mortality ^b	Condition factor ^c	
		48 h	7 days
0	10.0 $\pm$ 2.9	9.3 $\pm$ 0.2	9.5 $\pm$ 0.2
2	11.7 $\pm$ 6.0	9.2 $\pm$ 0.2	10.3 $\pm$ 0.3
20	8.3 $\pm$ 4.4	9.9 $\pm$ 0.3	9.9 $\pm$ 0.2
200	8.3 $\pm$ 3.3	9.7 $\pm$ 0.2	9.9 $\pm$ 0.3
400	9.0 $\pm$ 3.2	9.7 $\pm$ 0.3	10.1 $\pm$ 0.4
600	7.9 $\pm$ 3.2	9.7 $\pm$ 0.4	10.2 $\pm$ 0.5

^aAdapted from Carlson et al. (2002).

^bCumulative percent mortality at 7 days post-BaP injection (n = 3 individual experiments; 20 - 25 fish per treatment group $\pm$ S.E.M.).

^cCondition factor (weight (g)/[length (mm)]³) $\times 10^{-6}$ at 48 h and 7 days post-BaP injection (n = 3 individual experiments; 20 - 25 fish per treatment group $\pm$ S.E.M.)

Table 3.

Effects of benzo[a]pyrene (BaP) upon cell yield and viability in the immune organs of Japanese medaka 48 h post-injection^a

BaP concentration ($\mu\text{g BaP/g BW}$)	Thymic cellularity ^b		Kidney		Spleen	
	48 h	7 days	Cell yield ^c	Cell viability ^d	Cell yield	Cell viability
0	2606.6 $\pm$ 144.8	2774.9 $\pm$ 271.3	10.0 $\pm$ 0.7	91.3 $\pm$ 2.4	6.5 $\pm$ 0.4	80.3 $\pm$ 4.5
2	2870.5 $\pm$ 324.5	2080.9 $\pm$ 147.4	10.3 $\pm$ 0.5	90.0 $\pm$ 2.8	6.8 $\pm$ 0.4	78.8 $\pm$ 4.6
20	2752.3 $\pm$ 204.9	2374.3 $\pm$ 146.3	9.4 $\pm$ 0.3	93.7 $\pm$ 2.3	5.9 $\pm$ 0.6	82.5 $\pm$ 3.1
200	2425.3 $\pm$ 146.9	1966.6 $\pm$ 237.4	9.9 $\pm$ 0.4	89.6 $\pm$ 1.8	6.5 $\pm$ 0.2	78.3 $\pm$ 5.1

^aTable adapted from Carlson et al. (2002).^bMean thymic cellularity (number of thymocytes /mm²) as analyzed by computer-assisted video microscopy at 2 and 7 days post-BaP injection (n = 3 - 7 fish $\pm$ S.E.M.).^cMean cell yield ($\times 10^5$) /fish at 2 days post-BaP injection (n = 4 individual experiments; 10 - 15 fish pooled per treatment group $\pm$ S.E.M.).^dMean cell yield ($\times 10^5$) /fish at 2 days post-BaP injection (n = 4 individual experiments; 10 - 15 fish pooled per treatment group $\pm$ S.E.M.).

3. *Kidney Phagocyte Intracellular Superoxide Production*

Levels of intracellular $\bullet\text{O}_2^-$ within PMA-stimulated kidney phagocytes were significantly lower in 200 μg BaP /g BW-exposed animals compared to fish receiving vehicle control, 2, or 20 μg BaP /g BW doses (Figure 6); PMA-stimulated intracellular $\bullet\text{O}_2^-$ levels from fish receiving either 2 or 20 μg BaP /g BW were not significantly different from vehicle control levels. Additionally, no BaP-related effects upon kidney cell unstimulated $\bullet\text{O}_2^-$ levels were observed at any dose.

4. *Splenic Lymphocyte Proliferation*

Both T- and B-lymphocyte proliferation were extremely sensitive to BaP exposure. Splenic lymphocytes recovered from fish exposed to 2, 20, or 200 μg BaP /g BW had significantly reduced Con A- and LPS-stimulated lymphoproliferative responses compared to the vehicle controls (Figure 7). No significant differences in lymphocyte proliferation were observed between any of the BaP exposure groups.

Since the MTT assay is an indirect method of assessing proliferation, cells were also obtained from splenic lymphocyte cultures 3 d following mitogen exposure and cell density /viability determined. No differences in either cell number or viability were observed between cultures from either the unstimulated group or any treatment

Figure 6. Effects of IP exposure to BaP upon medaka intracellular superoxide ($\bullet\text{O}_2^-$) production by kidney phagocytes. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW and sacrificed 48 h later for determination of PMA-stimulated or unstimulated $\bullet\text{O}_2^-$ production. Values represent the mean nmoles of $\bullet\text{O}_2^-$ produced / 6×10^5 cells / 60 min ($n = 3 - 4$ experiments $\pm$ S.E.M.). * Significantly different ($p \leq 0.05$) from PMA-stimulated vehicle control, 2, and 20 $\mu\text{g/g}$ BW treatment groups. Adapted from Carlson et al. (2002).

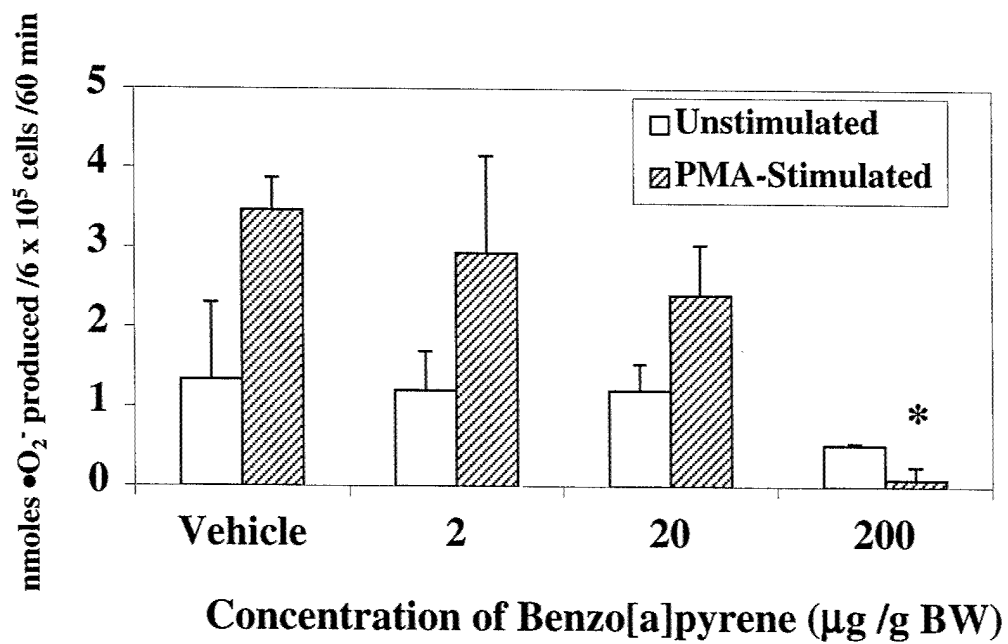
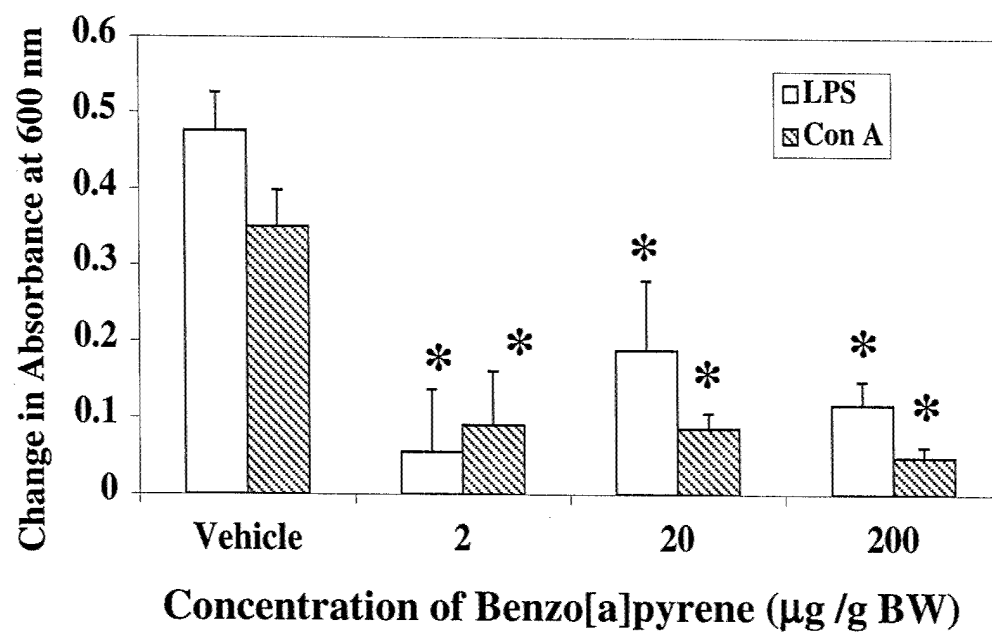


Figure 7. Effects of IP exposure to BaP upon mitogen-stimulated proliferation of splenic lymphocytes. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 μg /g BW and sacrificed 48 h later for determination of splenic B- and T-lymphocyte proliferation (as measured by an MTT assay) following *in vitro* exposure to either LPS or Con A, respectively. Values represent the mean change in absorbance at 600 nm (O.D. of mitogen-stimulated cells – O.D. of unstimulated cells) [$n = 3 - 5$ experiments $\pm$ S.E.M.]. * Significantly different from mitogen-matched vehicle control ($p \leq 0.05$). Adapted from Carlson et al. (2002).



group following LPS-stimulation. However, significantly fewer cells were recovered from cultures originally obtained from fish exposed *in vivo* to 20 and 200 µg BaP /g BW and stimulated *in vitro* with Con A as compared to vehicle control cells (Figure 8A). Con A-stimulated cultures from the 2 µg BaP /g BW treatment group possessed fewer cells than vehicle control, although the differences failed to reach statistical significance. No differences in cell viability were observed between any treatment group (Figure 8B).

5. *Plaque Forming Cell Assay*

Medaka exposed to either 20 or 200 µg BaP /g BW exhibited significantly lower numbers of PFCs compared to vehicle control (Figure 9); fish exposed to 200 µg BaP /g BW also demonstrated significantly fewer PFCs than fish receiving the lowest BaP concentration (2 µg /g BW). Only a modest reduction in PFC numbers ($P > 0.05$) was seen in the lowest BaP dose as compared to vehicle control.

6. *Host Resistance to Bacterial Challenge*

Mortality following bacterial challenge of fish receiving any BaP dose was not significantly different from vehicle control at 3 d post-challenge (Figure 10). However, fish injected with either 20 or 200 µg BaP /g BW had significantly higher mortality compared to vehicle control at 14 d post-challenge. Furthermore, at 7 d post-infection, only those fish exposed to 200 µg BaP /g BW had significantly reduced survival compared to vehicle controls. Of particular interest is the consistent presence

Figure 8. Effects of IP exposure to BaP upon mitogen-stimulated splenocyte density and viability. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW and sacrificed 48 h later for determination of *ex vivo* mitogen-stimulated splenic lymphocyte number and viability by hemacytometer counting and trypan blue exclusion, respectively. Values represent either (A) mean number of recovered cells ($\times 10^4$) /culture or (B) percent viability of cell cultures after 72 h incubation with either no mitogen, LPS (B-cell mitogen), or Con A (T-cell mitogen)[n = 3 experiments $\pm$ S.E.M.]. * Significantly different from mitogen-matched vehicle control ($p \leq 0.05$).

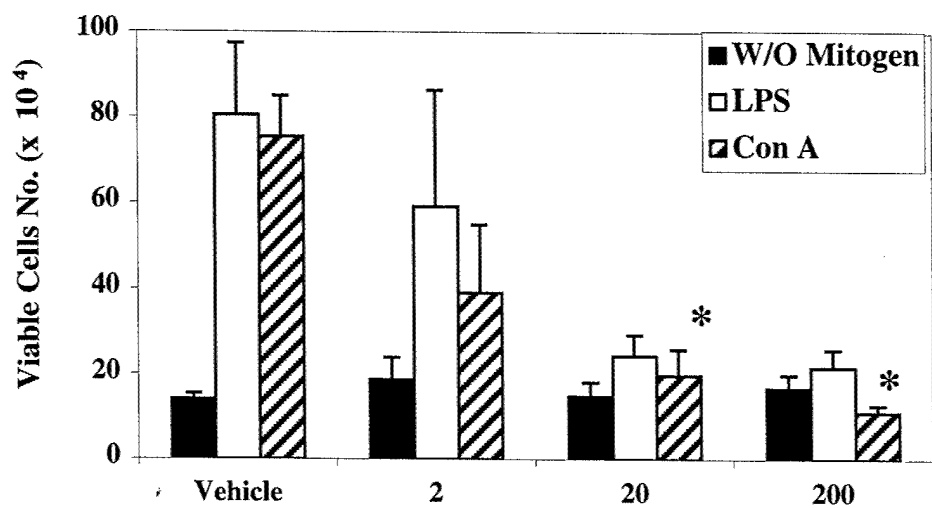
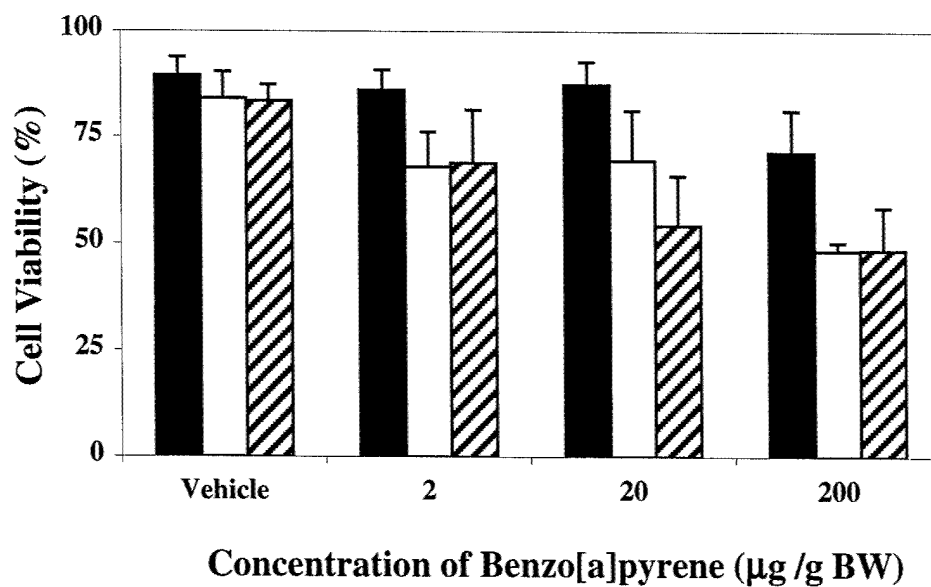
A**B**

Figure 9. Effects of IP exposure to BaP upon medaka plaque-forming cell (PFC) numbers. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW and immunized IP 48 h post-injection with sheep red blood cells (SRBCs) for later determination of kidney PFC numbers. Values represent the mean number of plaques / 2×10^6 kidney cells ($n = 3 - 5$ experiments $\pm$ S.E.M.). * Significantly different from vehicle control ($p \leq 0.05$). # Significantly different from the 2 μg BaP /g BW exposure group ($p \leq 0.05$). Adapted from Carlson et al. (2002).

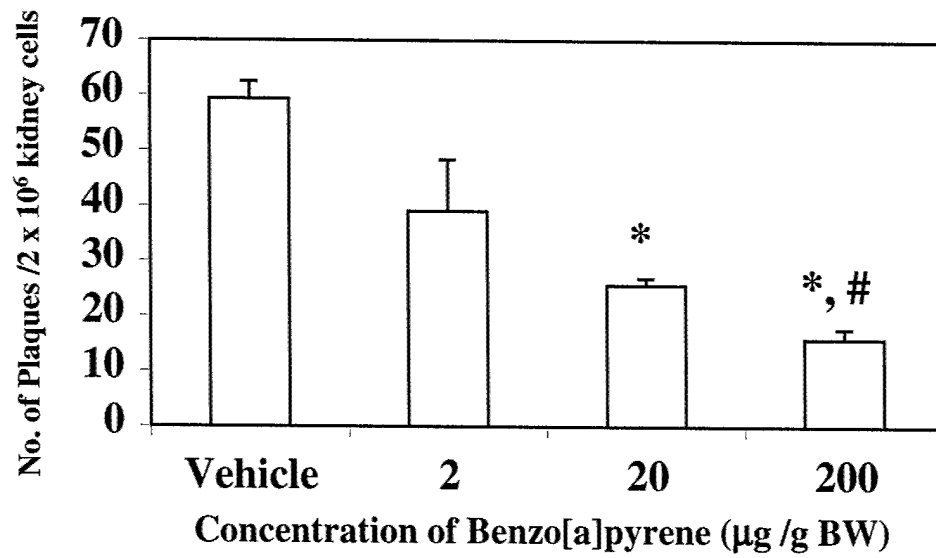
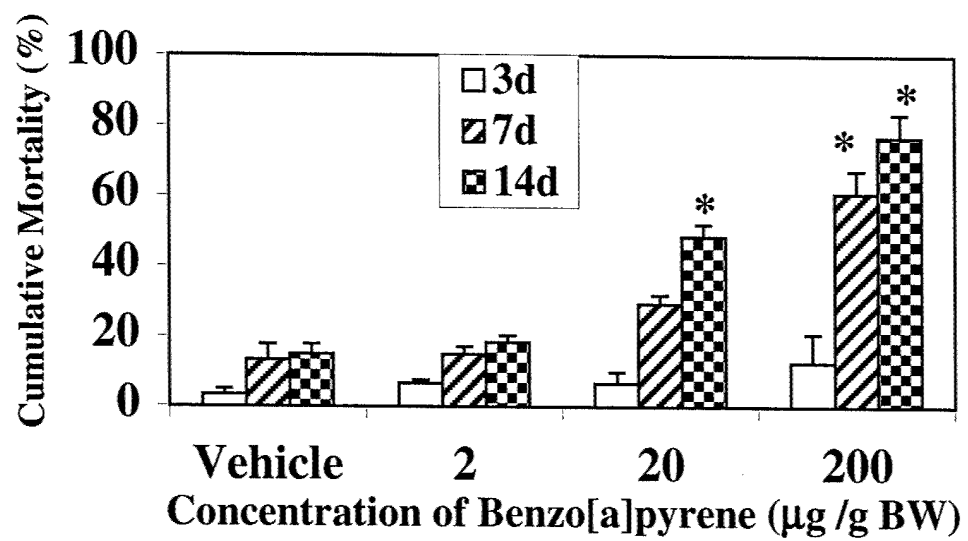


Figure 10. Effects of IP exposure to BaP upon host resistance against bacterial challenge. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW, infected with 2×10^6 *Y. ruckeri* 48 h later, and host survival monitored for up to 14 d. Values represent the percent cumulative mortality at 3, 7, and 14 d post-infection ($n = 3$ individual experiments of 20 – 25 fish each $\pm$ S.E.M.). *Significantly different from time-matched vehicle control ($p \leq 0.05$). Adapted from Carlson et al. (2002).



of pin-point red petechiae randomly distributed along the bodies of bacteria-exposed fish that received 200 µg BaP /g BW injections prior to *Y. ruckeri* challenge. Such lesions were not consistently observed in other treatment groups.

B. SPECIFIC AIM 2

1. BaP Metabolites in Bile

Significantly higher fixed fluorescence (FF) /µg bile protein was observed in bile samples taken from fish 48 h post-injection of 200 µg BaP /g BW compared to those from vehicle-injected control animals or those from the two lower BaP exposure groups (Figure 11). Fixed-fluorescence in bile samples from fish receiving either 2 or 20 µg BaP /g BW were no different than vehicle control.

2. Microsomal CYP1A Protein Expression in Liver, Kidney, & Spleen

Initial Western blot analysis of liver microsomal samples from fish receiving 200 µg BaP /g BW revealed that BaP exposure resulted in the induction of a single, 52 kDa protein that was immunoreactive with monoclonal antibodies C10-7 and 1-12-3 (Figure 12). CYP1A ELISA analysis of microsomal samples prepared from flash-frozen livers revealed that samples from fish exposed to either 20 or 200 µg BaP /g BW exhibited significantly greater hepatic CYP1A protein levels compared to vehicle controls at 48 h post-injection (Figure 13A). Hepatic CYP1A protein levels in fish receiving 2 µg BaP /g BW were no different than the vehicle control, 20, or 200 µg BaP /g BW treatment groups.

Figure 11. Effects of IP exposure to BaP upon the relative amounts of fluorescent aromatic compounds (FACs) in medaka bile. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 μg /g BW prior to determination of fixed fluorescence (FF; 380 nm excitation /430 nm emission) in bile collected 48 h post-injection. Fluorescence (under these conditions) correlates with peak absorbance of 5-ring aromatic compounds (i.e., BaP). Values represent the mean FF / μg of bile protein ($n = 3 - 4$ experiments $\pm$ S.E.M.). * Significantly different from vehicle control, 2, and 20 μg BaP /g BW exposure groups ($p \leq 0.05$).

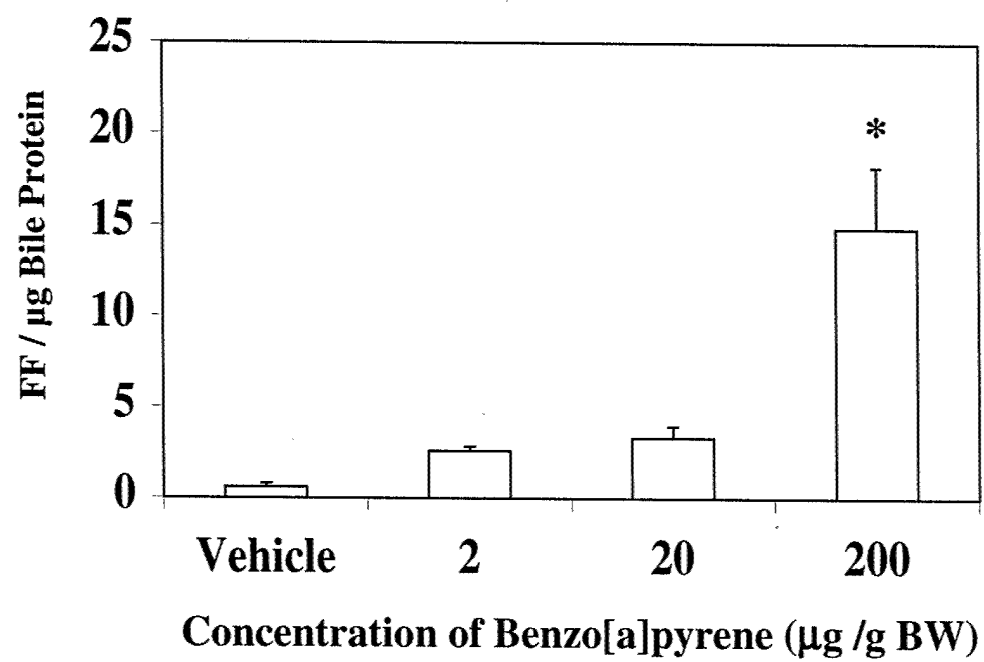
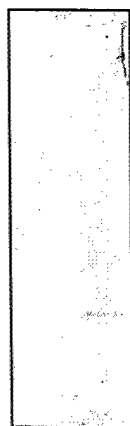


Figure 12. Western blot analysis of hepatic microsomal CYP1A protein levels 48 h post-BaP injection. Medaka were injected IP with either vehicle control or BaP at 200 $\mu\text{g/g}$ BW and microsomes prepared from pooled livers (3 organs /sample). Western blotting was performed following SDS-PAGE separation of microsomal proteins on 10% polyacrylamide gels. The primary antibodies used to probe medaka CYP1A proteins were either (A) monoclonal antibody C10-7 (specific for a conserved region of rainbow trout CYP1A1) or (B) monoclonal antibody 1-12-3 (specific for scup CYP1A1 protein). Arrows point towards a single immunoreactive band (for either antibody) representing a putative 52 KDa CYP1A protein (only visible in samples from 200 μg BaP /g BW-exposed medaka).

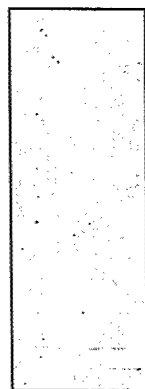
A



Vehicle

200 µg BaP /g BW

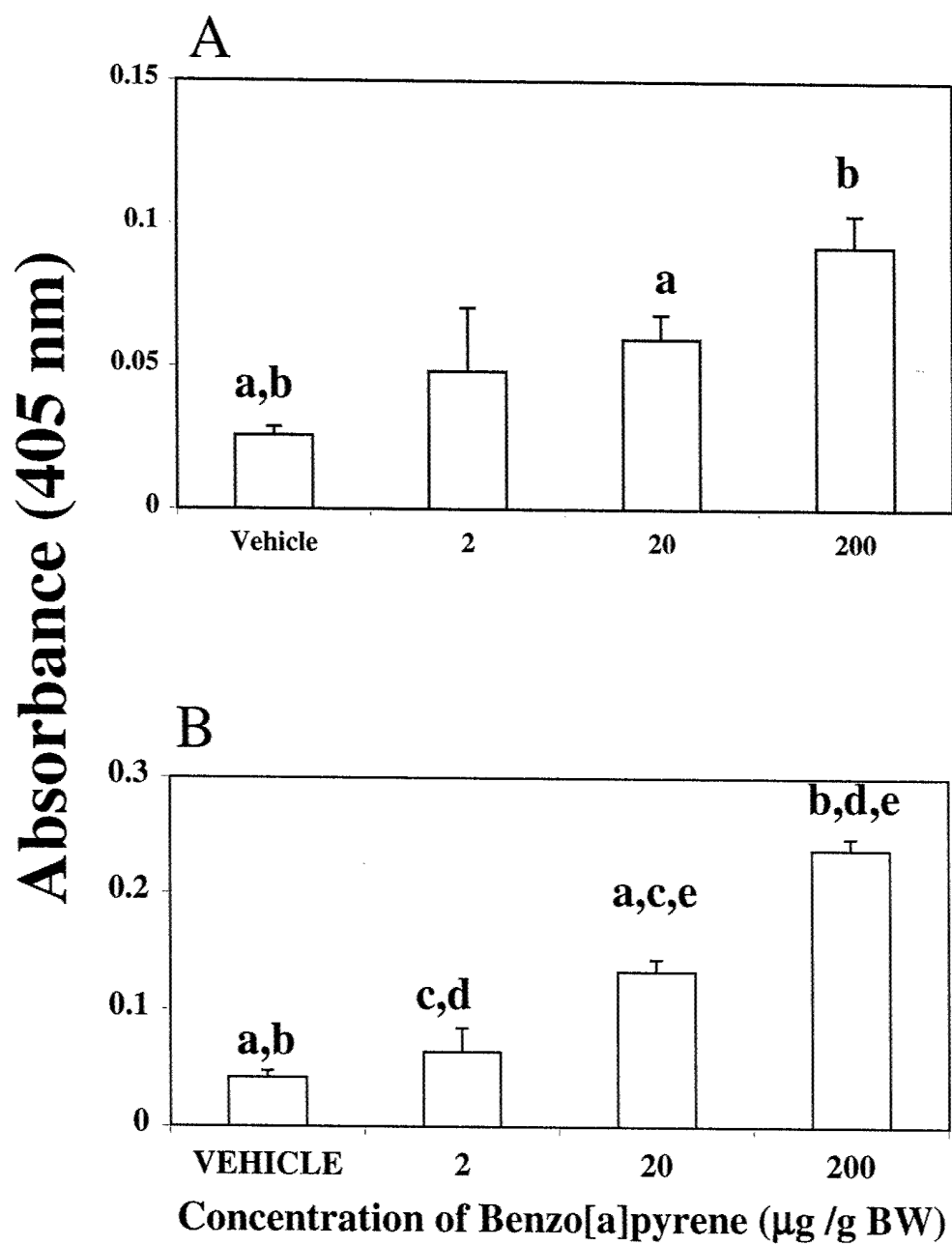
B



Vehicle

200 µg BaP /g BW

Figure 13. Enzyme-linked immunosorbant assay (ELISA) analysis of hepatic microsomal CYP1A protein levels 48 h post-BaP injection. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to liver microsome preparation. Relative levels of hepatic microsomal CYP1A protein were determined by ELISA using monoclonal C10-7 antibody as a probe. Values represent the mean absorbance at 405 nm for microsomal samples prepared from either previously-frozen livers (A) or fresh tissue (B) [n = 3 - 4 independent experiments consisting of 3 - 5 microsomal samples prepared from individual livers $\pm$ S.E.M.]. ^{a-c} Data with the same letter are significantly different from each other ($p \leq 0.05$).



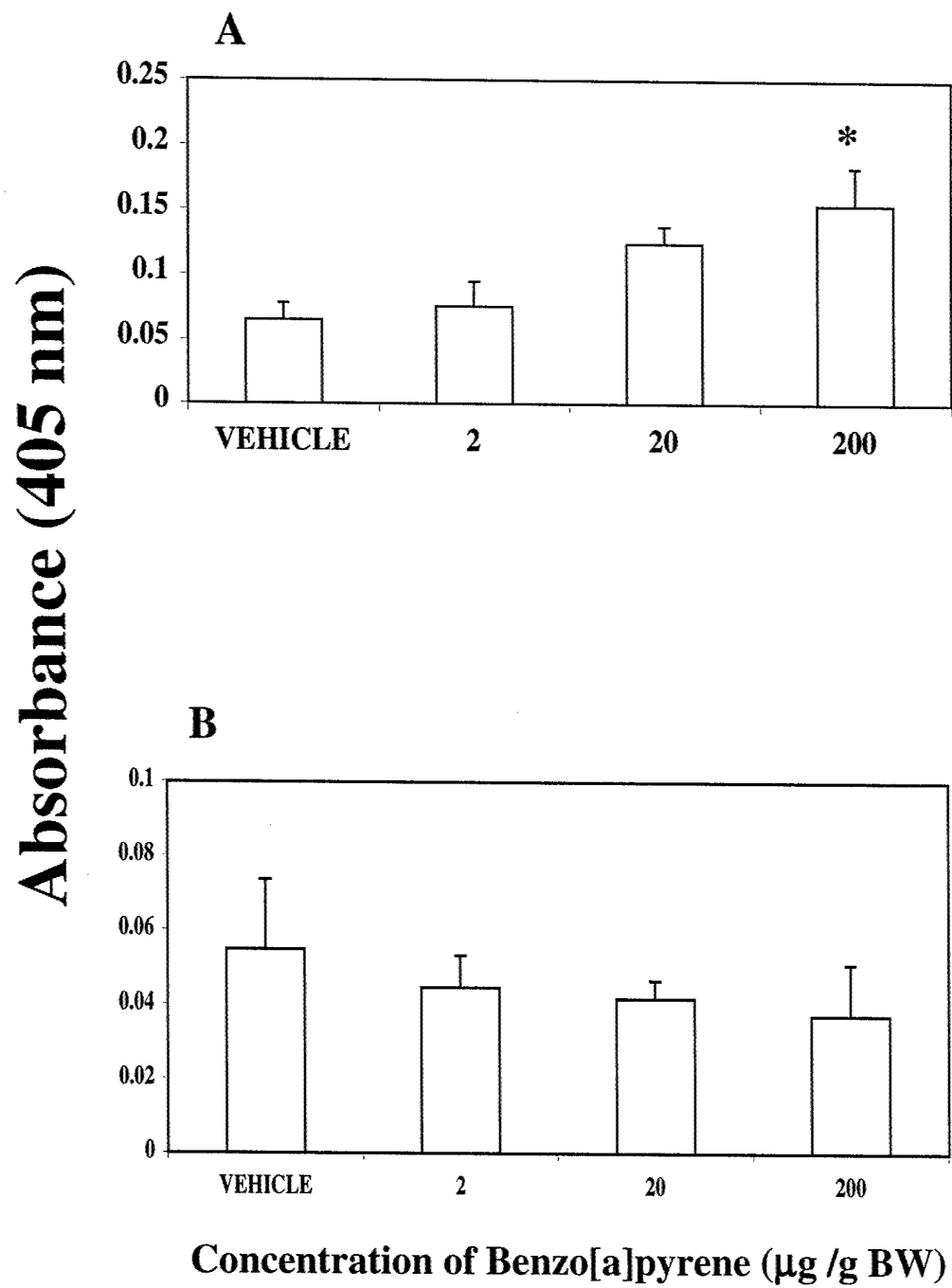
Hepatic CYP1A microsomal protein levels from fresh rather than flash-frozen livers were elevated in fish treated with 20 and 200 µg BaP /g BW compared to the vehicle control and 2 µg BaP /g BW treatment group (Figure 13B). In addition, liver microsomal CYP1A expression at the highest BaP dose was significantly greater than that in the 20 µg BaP /g BW treatment group. Although flash-frozen microsomal preparations appeared to possess relatively lower CYP1A protein levels (as compared to freshly-prepared samples), the mean CYP1A protein levels obtained following each treatment for both preparations correlated significantly with each other ($R^2 = 0.946$; $p \leq 0.05$).

Analyses of microsomal CYP1A expression in organs other than liver utilized microsomes prepared from fresh tissue. Examination of kidney microsomes obtained 48 h post-injection demonstrated that CYP1A protein levels were significantly greater in fish exposed to 200 µg BaP /g BW than that observed in either the vehicle control or 2 µg BaP /g BW treatment group (Figure 14A); neither of the two lower BaP doses significantly induced CYP1A protein (compared to the vehicle control). Treatment with BaP had no effect upon CYP1A expression by splenic microsomes (Figure 14B).

3. *Microsomal CYP1A Enzyme Activity in Liver, Kidney, & Spleen*

Initial studies utilizing microsomes prepared 48 h post-injection from flash-frozen medaka livers demonstrated that both the 20 and 200 µg BaP /g BW treatment groups had significantly increased EROD activity as compared to either the vehicle

Figure 14. ELISA analysis of kidney and spleen microsomal CYP1A protein levels 48 h post-BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to preparation of microsomes from pooled (3-10 organs /treatment group) kidney or spleen samples. Relative levels of microsomal CYP1A protein were determined by ELISA using monoclonal C10-7 antibody as a probe. Values represent the mean absorbance at 405 nm for microsomal samples prepared from either kidney (A) or spleen (B) [n = 3 - 4 independent experiments $\pm$ S.E.M.]. * Significantly different from vehicle control and the 2 μg BaP /g BW exposure group ($p \leq 0.05$).



control or the lowest BaP exposure group (Figure 15A). Liver microsomes prepared from fresh organs demonstrated significantly higher EROD activities in the 20 and 200 µg BaP /g BW exposure groups (compared to either vehicle control or the 2 µg BaP /g BW exposure group; Figure 15B); fish treated with 200 µg BaP /g BW also demonstrated significantly greater hepatic EROD activity in freshly prepared microsomes than did the 20 µg BaP /g BW dose group. While it appears that flash-freezing microsomes decreased EROD activity compared to freshly-prepared preparations, a positive correlation ($p \leq 0.05$) was seen upon comparison of mean EROD activities for each treatment group prepared by different methods ($R^2 = 0.9808$). Significantly higher renal EROD activity was observed in kidney microsomes from fish receiving 200 µg BaP /g BW compared to the vehicle control, 2, or 20 µg BaP /g BW exposure groups (Figure 16); EROD activity in both the 2 and 20 µg BaP /g BW treatment groups were similar to vehicle control levels. Microsomal preparations of pooled spleen samples from all BaP exposure groups contained no detectable levels of EROD activity (data not shown).

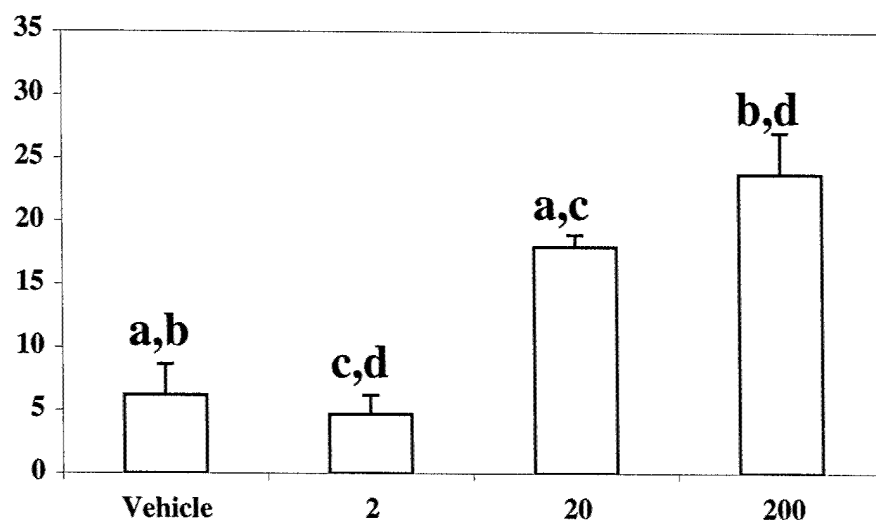
4. CYP1A Protein Expression in Specific Cell/Tissue Types within Liver, Kidney, & Spleen

Immunohistochemical analysis of hepatic CYP1A expression revealed basal CYP1A expression by hepatocytes (Panel 2). Exposure of fish to 200 µg BaP /g BW significantly induced hepatic CYP1A expression compared to vehicle control (Table 4). A modest but insignificant level of CYP1A induction was also observed in the 20

Figure 15. Ethoxyresorufin *O*-deethylase (EROD) activity of hepatic microsomes 48 h post-BaP injection. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 µg /g BW prior to liver microsome preparation. Levels of hepatic EROD activity were determined by a microtitre plate EROD assay. Values represent the mean pmoles of resorufin produced /min /mg protein for microsomal samples prepared from either frozen livers (A) or fresh tissue (B) [n = 3 - 4 independent experiments consisting of 3 – 5 microsomal samples prepared from individual livers ± S.E.M.]. ^{a-c} Data with the same letter are significantly different from each other ($p \leq 0.05$).

pmoles resorufin /min /mg protein

A



B

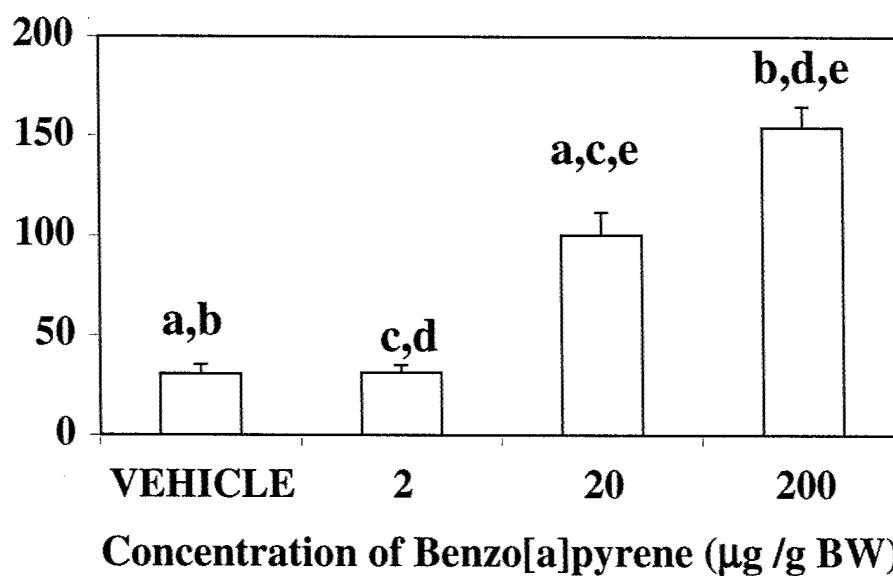
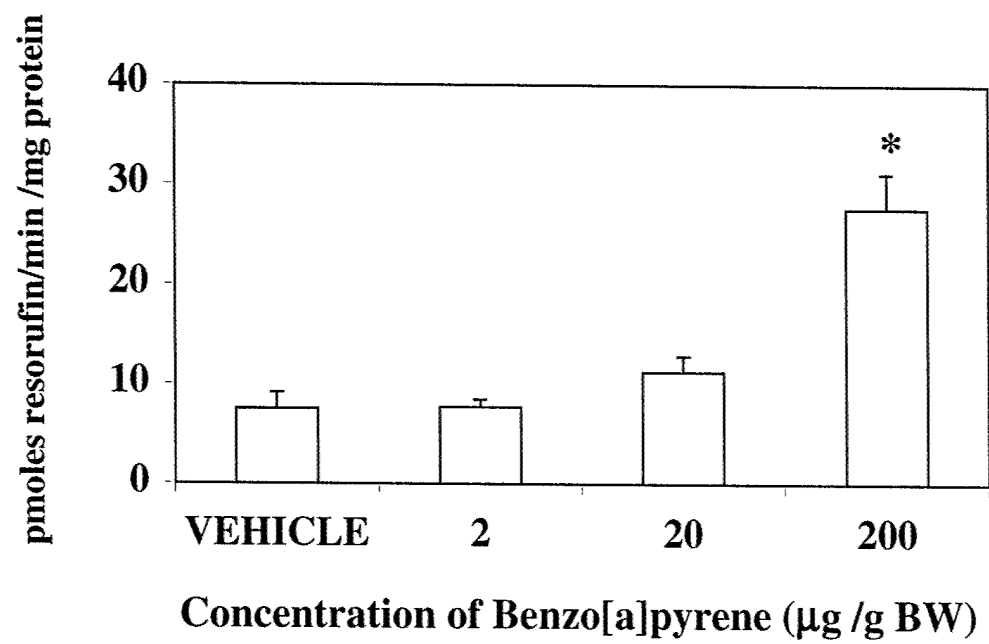


Figure 16. EROD activity of kidney microsomes 48 h post-BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to preparation of microsomes from pooled (3-10 organs /treatment group) kidney samples. Levels of kidney microsomal EROD activity were determined by a microtitre plate EROD assay. Values represent the mean pmoles of resorufin produced /min /mg protein ($n = 4$ independent experiments $\pm$ S.E.M.). * Significantly different from vehicle control, 2, and 20 $\mu\text{g BaP/g BW}$ exposure groups ($p \leq 0.05$).



Panel 2. Immunohistochemical localization of CYP1A in medaka liver 48 h following BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to histological processing of tissue at 48 h post-injection. (A) Vehicle control liver displaying weak staining for CYP1A within hepatocytes. (B) 20 $\mu\text{g BaP/g BW}$ – exposed liver displaying moderate staining for CYP1A within hepatocytes. (C) 200 $\mu\text{g BaP/g BW}$ – exposed liver displaying intense staining for CYP1A within hepatocytes. (original magnifications: A - C, 400X)

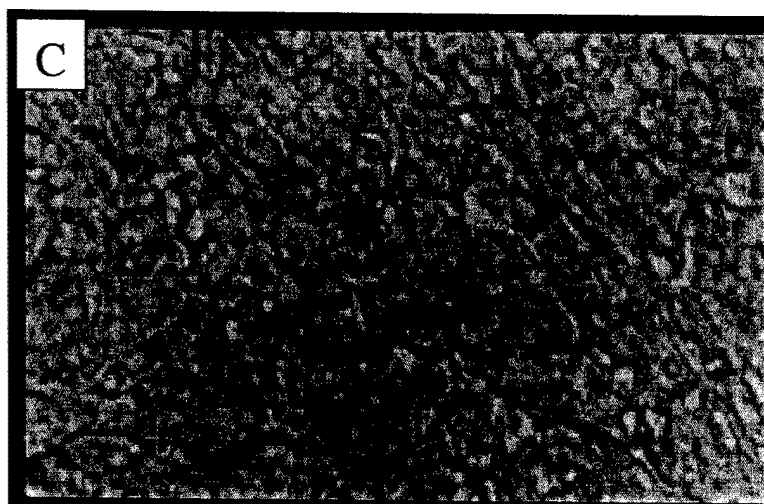
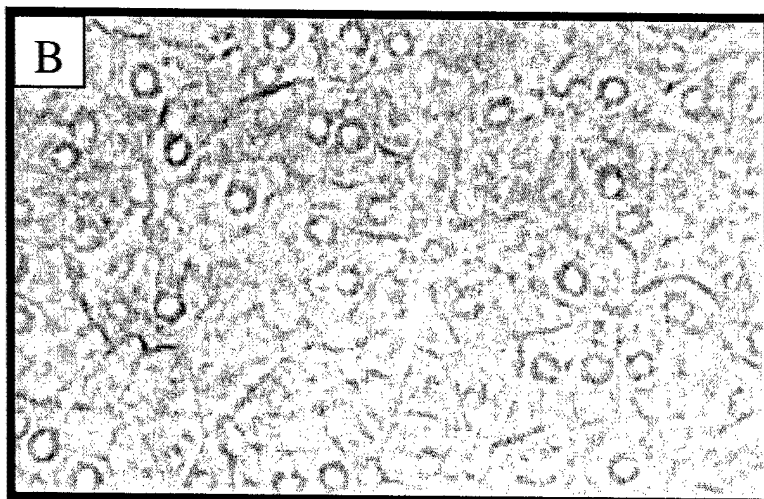
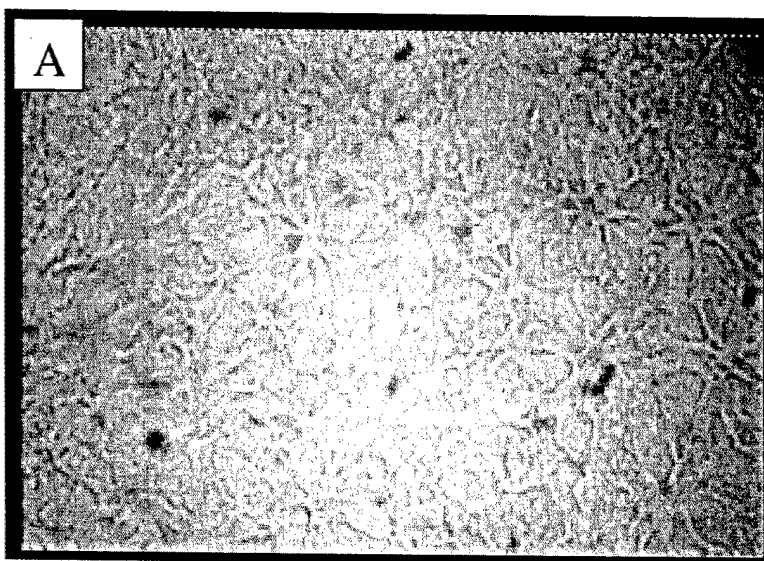


Table 4.

Effects of benzo[a]pyrene (BaP) upon CYP1A immunohistochemistry (IHC) in medaka hepatocytes, kidney tubules and hematopoietic tissue, and splenic endothelia 48 h post-injection

BaP concentration ($\mu\text{g/g BW}$)	CYP1A IHC Index ^a			
	Liver	Kidney		Spleen
	Hepatocytes	Tubules	Hematopoietic Tissue	Endothelia
0	$4.67 \pm 0.48^*$	$3.37 \pm 0.38^{*,\#}$	ND ^b	ND
2	$4.86 \pm 0.77^\#$	$3.08 \pm 0.36^{@,\%}$	$0.08 \pm 0.08^*$	$0.50 \pm 0.50^*$
20	$6.04 \pm 0.46^{@}$	$6.50 \pm 0.61^{*,@,\$}$	$1.28 \pm 0.49^\#$	3.17 ± 1.30
200	$9.13 \pm 0.84^{*,\#,@}$	$9.63 \pm 0.74^{\#,\%,\$}$	$3.74 \pm 0.52^{*,\#}$	$6.06 \pm 0.87^*$

^aMean CYP1A IHC Index determined 48 h post-BaP injection (n = 3 individual experiments; 3 - 10 fish per treatment group $\pm$ S.E.M.).

^b ND = no detectable CYP1A staining (CYP1A IHC Index = 0).

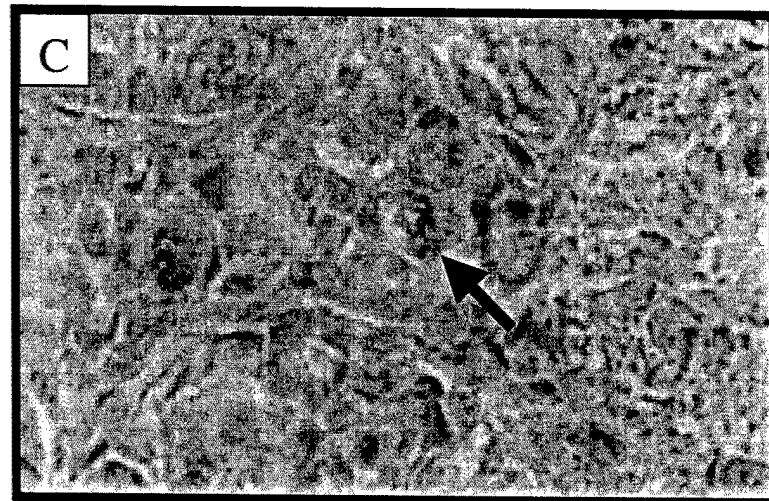
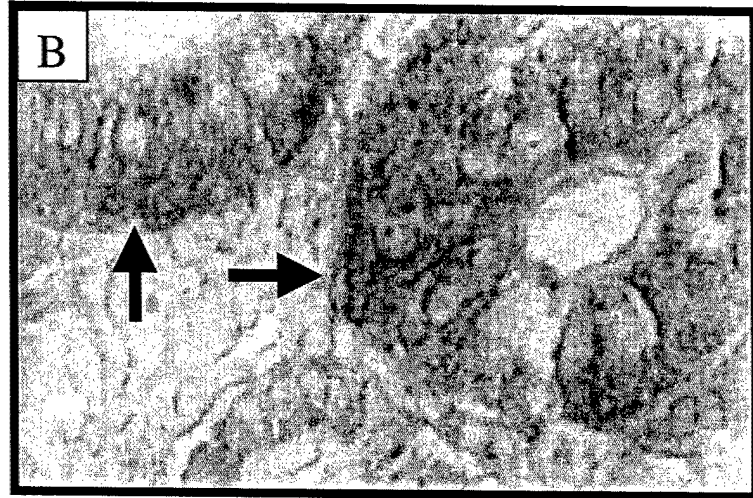
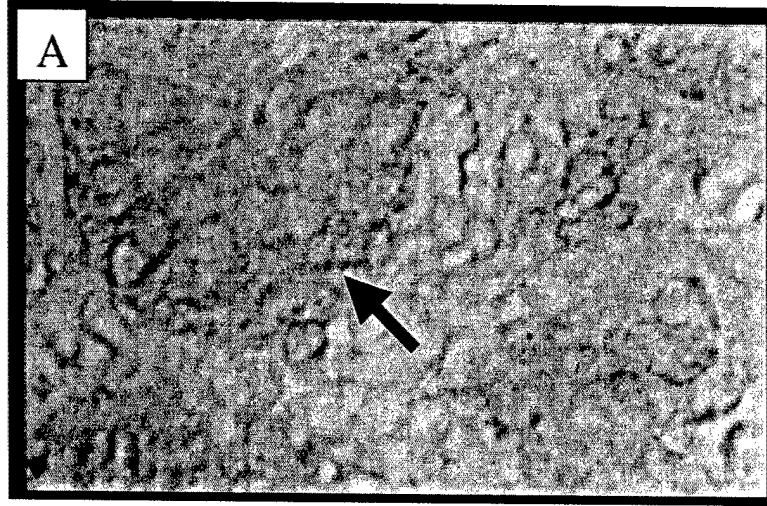
*,#,@,%,\$ Within a particular tissue, data with the same symbol are significantly different from each other ($p \leq 0.05$).

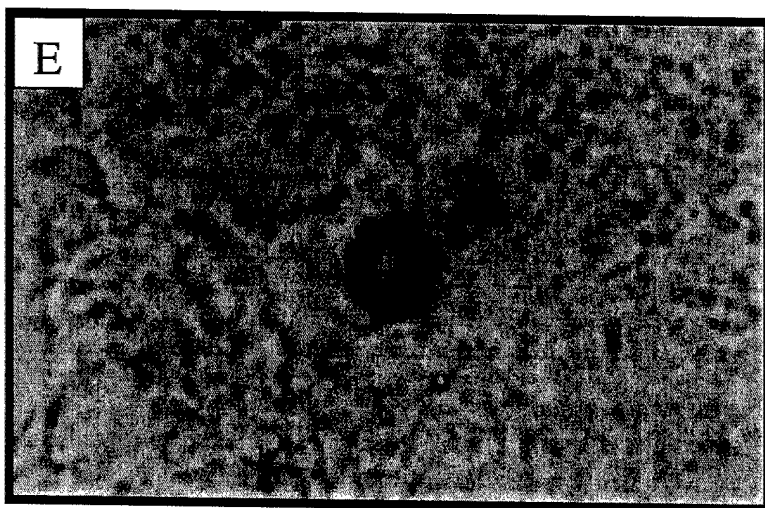
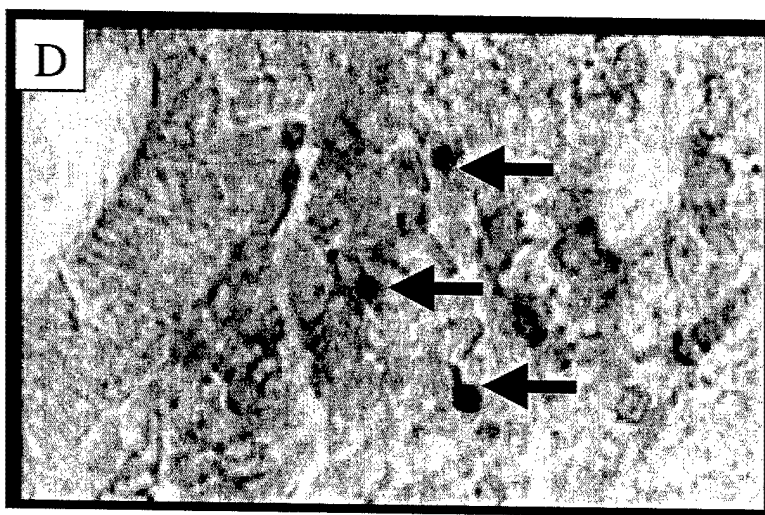
$\mu\text{g BaP/g BW}$ exposure group. Hepatocyte CYP1A expression in fish exposed to the highest BaP concentration was also significantly greater than observed at the two lower doses. In addition to hepatocytes, CYP1A expression was also rarely observed in the hepatic endothelia.

Immunohistochemical evaluation of medaka renal tubules revealed that this tissue possessed basal CYP1A expression in some fish (Table 4). Moreover, both 20 and 200 $\mu\text{g BaP/g BW}$ exposure groups had significantly greater CYP1A IHC Indices than either the vehicle control or 2 $\mu\text{g BaP/g BW}$ treatment group (Panel 3A and B; Table 4). CYP1A IHC Indices for the highest BaP dose were also significantly greater than those observed for the 20 $\mu\text{g BaP/g BW}$ treatment group. CYP1A expression in renal tubules of fish receiving 2 $\mu\text{g BaP/g BW}$ was similar to that observed in the vehicle control. Basal and induced expression of renal tubular CYP1A was most evident in the proximal tubules; however, expression in distal tubules was also observed in samples with the highest CYP1A IHC Indices. BaP-induced expression was also occasionally observed in kidney endothelia and interrenal tissue and, thus, CYP1A IHC Indices were not calculated for these particular tissues (Panel 3C and F).

Of particular interest was the BaP-induced expression of CYP1A protein in a subpopulation of mononuclear cells within kidney hematopoietic tissues (Panel 3D and E). Significantly higher CYP1A IHC Indices for kidney hematopoietic tissue were observed in fish exposed to 200 $\mu\text{g BaP/g BW}$ compared to the 2 and 20 $\mu\text{g BaP/g BW}$ treatment groups (Table 4). Expression of CYP1A within kidney mononuclear

Panel 3. Immunohistochemical localization of CYP1A in medaka kidney 48 h following BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to histological processing of tissue at 48 h post-injection. (A) Vehicle control kidney displaying weak staining for CYP1A within renal tubules (arrow). (B) 200 $\mu\text{g BaP/g BW}$ –exposed kidney displaying moderate staining for CYP1A within renal tubules (arrow). (C) 20 $\mu\text{g BaP/g BW}$ –exposed kidney displaying moderate staining for CYP1A within vascular endothelia (arrow). (D and E) 200 $\mu\text{g BaP/g BW}$ –exposed kidney displaying intense staining for CYP1A within mononuclear cells (arrows) of the hematopoietic tissue. (F) 200 $\mu\text{g BaP/g BW}$ –exposed kidney displaying intense staining for CYP1A within interrenal tissue (arrow). (original magnifications: A – D, F, 400X; E, 1000X)





cells was very rarely observed in tissue from fish exposed to the lowest BaP dose and completely absent in the vehicle control animals.

Splenic endothelia were also examined by IHC for expression of CYP1A protein. CYP1A expression was significantly greater in splenic endothelia from fish exposed to the highest BaP dose compared to those exposed to the lowest BaP concentration (Panel 4; Table 4). Fish exposed to 20 µg BaP /g BW only demonstrated CYP1A IHC Indices only modestly greater than the lowest BaP dose, while vehicle control medaka completely lacked CYP1A staining in splenic endothelia

5. *CYP1A Protein Expression in Isolated Kidney Mononuclear Cells*

Analysis of non-specific esterase activity for glass-adherent and non-adherent mononuclear cells following BaP exposure revealed no BaP-induced changes in the percentage of esterase positive cells for either cell population (Figure 17). While only a relatively low percentage (17 – 23%) of non-adherent cells expressed non-specific esterase activity, a significantly higher percentage (79 – 87%) of glass adherent cells were esterase-positive.

Significantly more glass-adherent and non-adherent isolated kidney mononuclear cells from fish exposed to the highest BaP dose expressed CYP1A protein compared to those from the vehicle control or the lowest BaP dose (Panel 5; Figure 18A). In addition, a significantly higher percentage of adherent cells from fish exposed to the highest BaP dose expressed CYP1A compared to adherent cells from fish injected with a ten-fold lower concentration. Fish exposed to either 2 or 20 µg

Panel 4. Immunohistochemical localization of CYP1A in medaka spleen 48 h following BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to histological processing of tissue at 48 h post-injection. (A) Vehicle control spleen displaying no staining for CYP1A; arrow = vascular endothelium (B) 200 μg BaP /g BW –exposed spleen displaying moderate staining for CYP1A within vascular endothelia; arrow = vascular endothelium. (original magnifications: A - B, 400X)

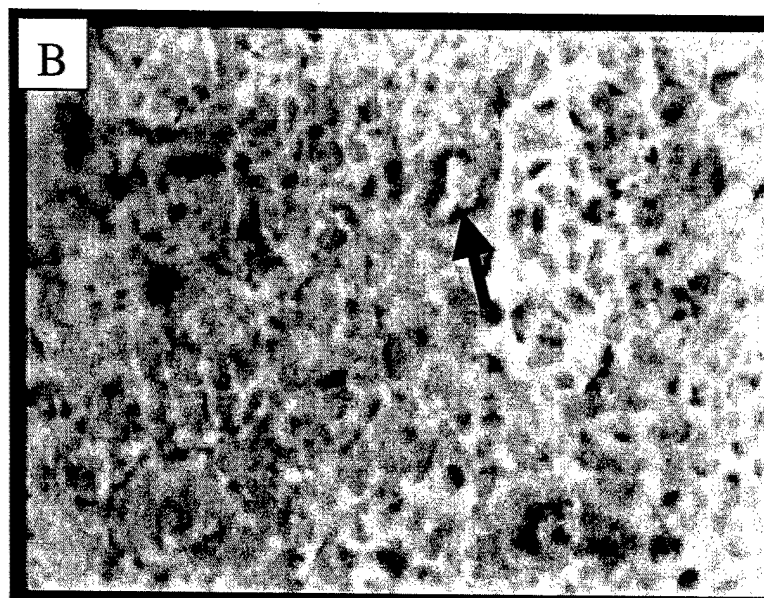
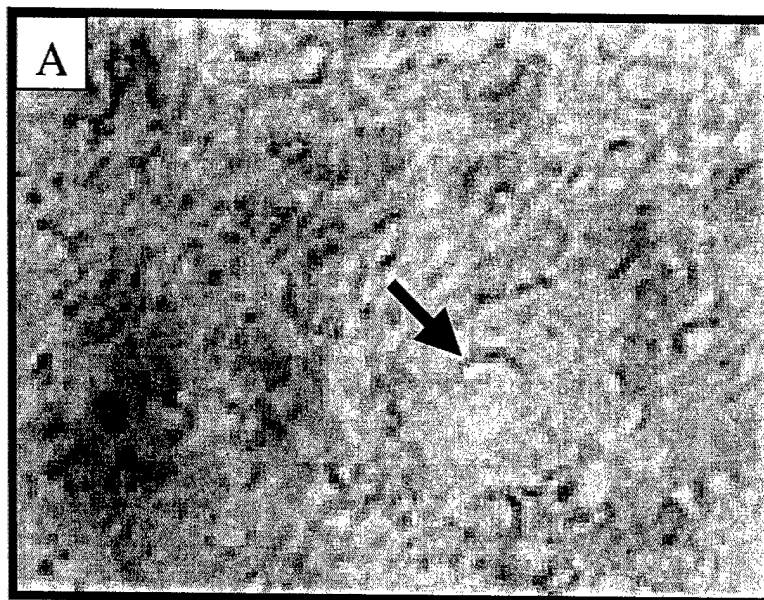
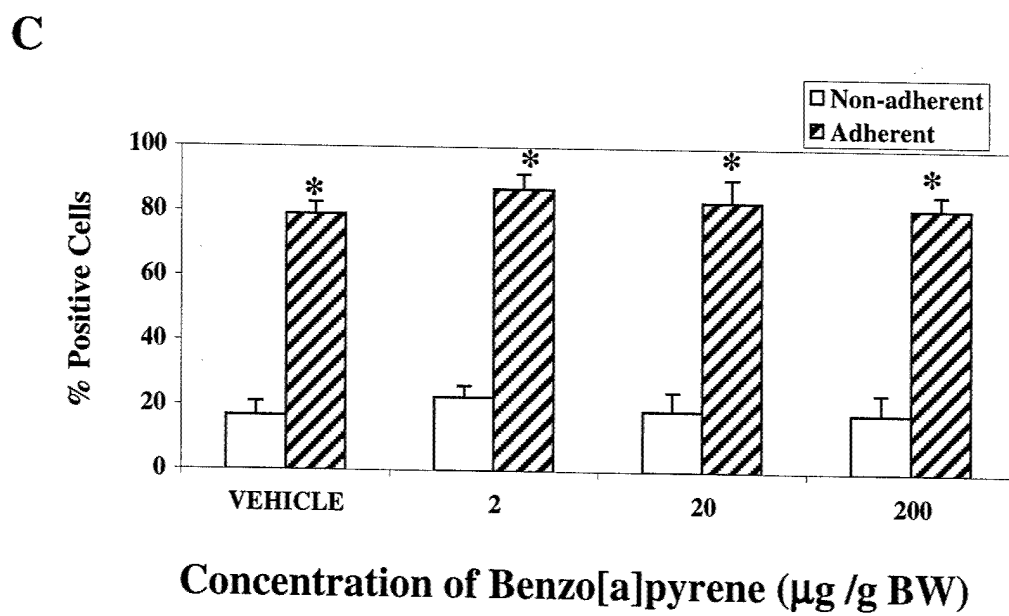
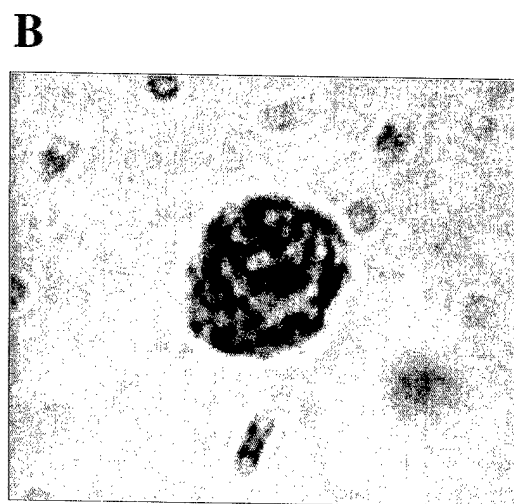
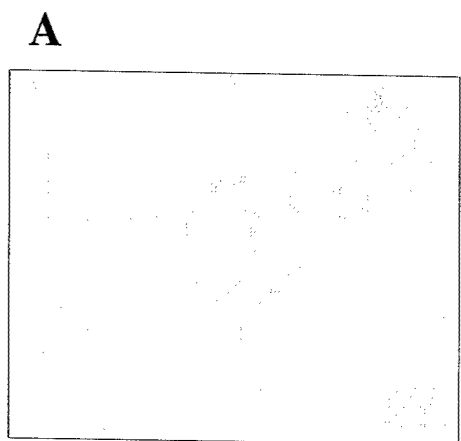
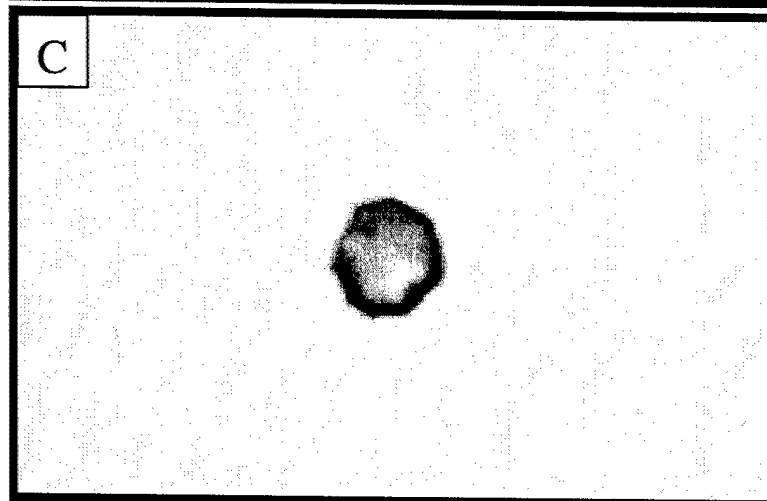
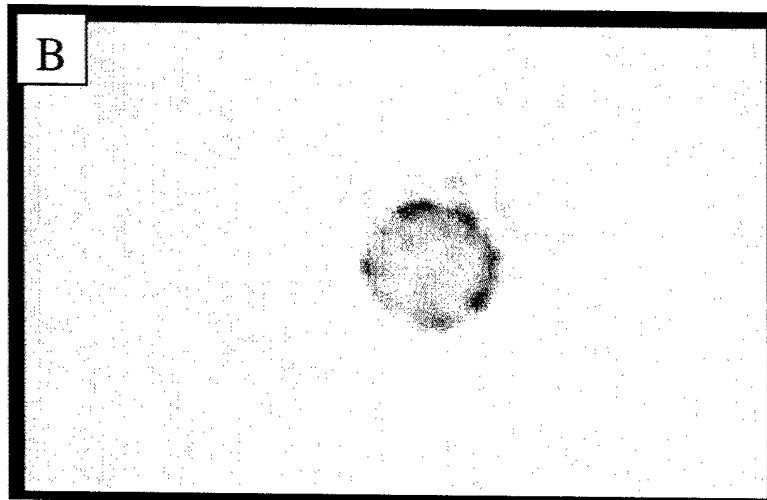
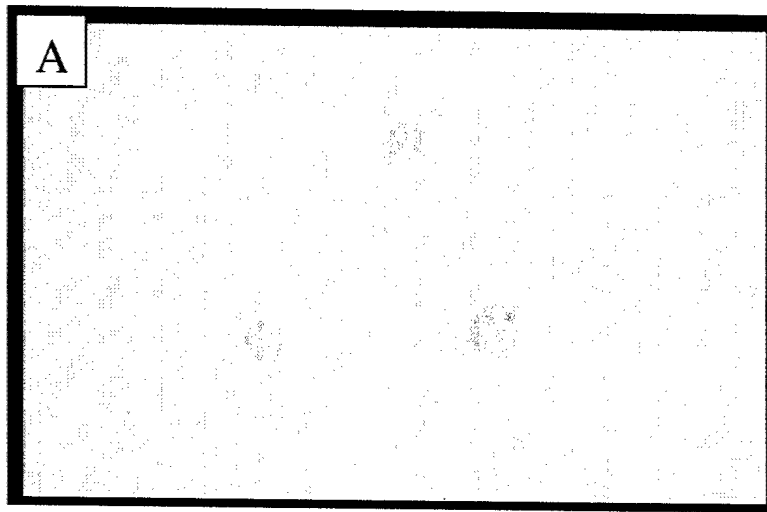


Figure 17. Non-specific esterase activity in kidney mononuclear cells isolated 48 h following BaP injection. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to isolation of kidney mononuclear cells. Non-specific esterase activity was assessed for glass-adherent and non-adherent mononuclear cells by incubation of cells with α -naphthyl acetate and stable diazonium salt. Esterase-negative cells (A) lacked any cytoplasmic granulation, while cells staining positive for esterase activity (B) demonstrated black granulation within the cellular cytoplasm. (C) One hundred cells per treatment were scored to obtain a mean percentage of esterase-positive adherent and non-adherent cells ($n = 3$ independent experiments $\pm$ S.E.M.). *Significantly different from non-adherent cells ($p \leq 0.05$). Photomicrographs are at a 1000 X magnification.



Panel 5. Immunocytochemical localization of CYP1A in isolated kidney mononuclear cells 48 h following BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g BW}$ prior to isolation of kidney mononuclear cells. Expression of CYP1A protein was assessed for both glass adherent and non-adherent mononuclear cells using CYP1A immunocytochemistry (ICC). (A) Vehicle control-exposed non-adherent cells displaying no staining for CYP1A. (B) 20 $\mu\text{g BaP/g BW}$ -exposed non-adherent cell displaying moderate staining for CYP1A. (C) 200 $\mu\text{g BaP/g BW}$ -exposed non-adherent cell displaying intense staining for CYP1A. (D) Vehicle control -exposed adherent cell displaying no staining for CYP1A. (E) 20 $\mu\text{g BaP/g BW}$ -exposed adherent cell displaying moderate staining for CYP1A. (F) 200 $\mu\text{g BaP/g BW}$ -exposed adherent cell displaying intense staining for CYP1A. (original magnifications: A, 400X; B - F, 1000X)



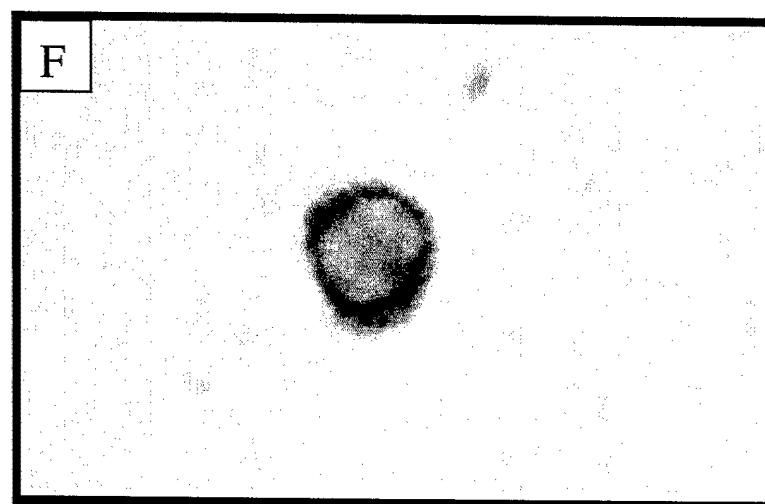
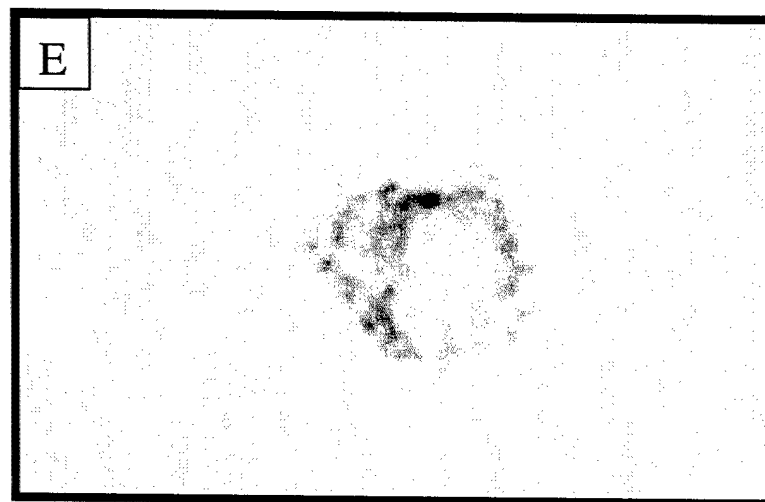
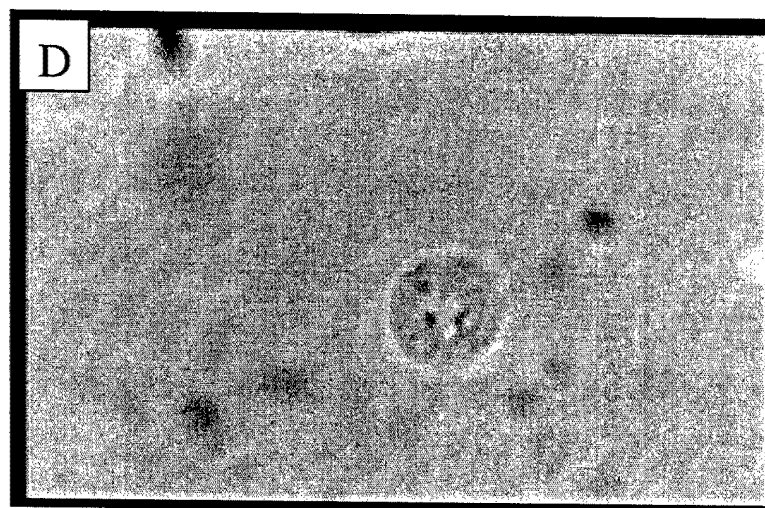
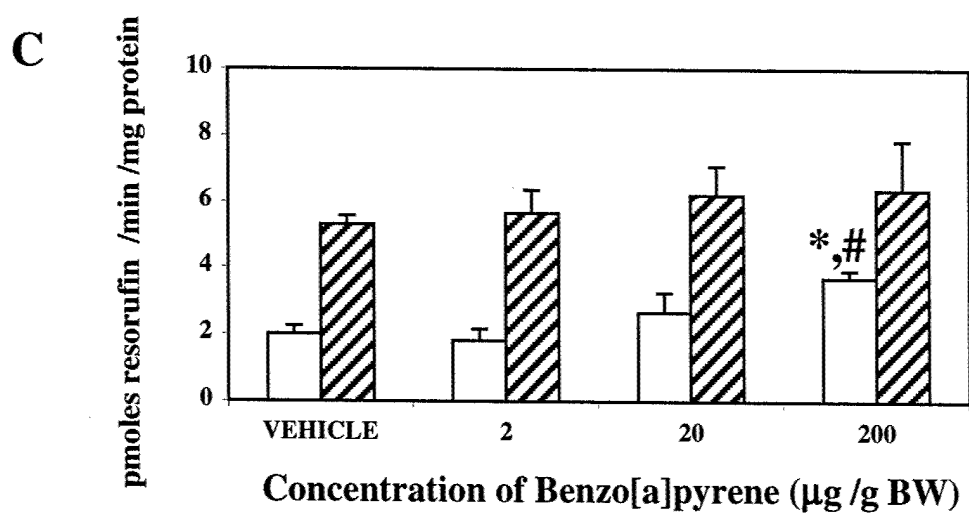
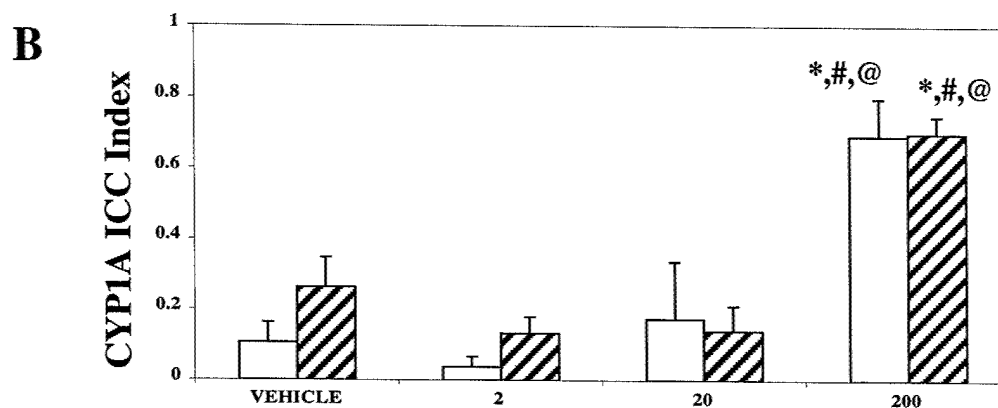
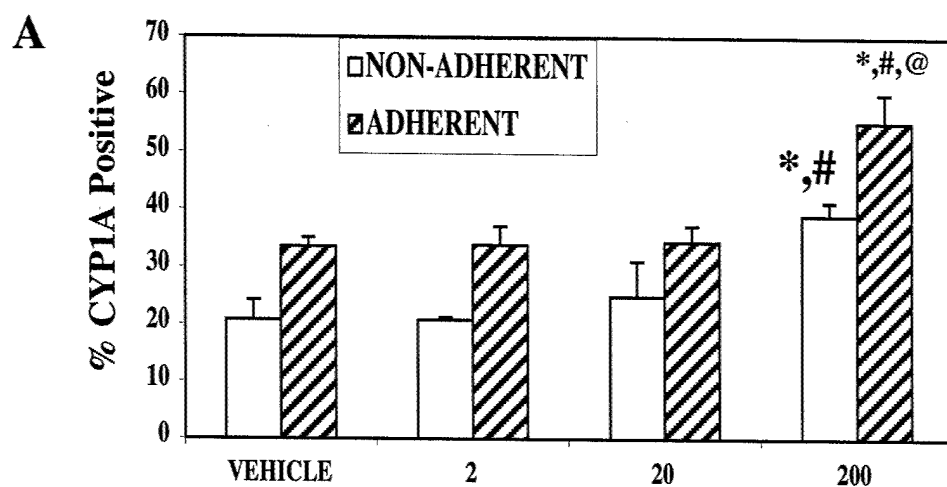


Figure 18. Analysis of CYP1A protein expression and activity in kidney mononuclear cells isolated 48 h post-BaP injection. Medaka were injected IP with either vehicle control or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to isolation of kidney mononuclear cells. CYP1A protein expression was assessed for glass-adherent and non-adherent mononuclear cells by CYP1A immunocytochemistry (ICC). Data was expressed as either (A) the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; $n = 3$ experiments $\pm$ S.E.M.), or (B) mean relative frequency of cells expressing moderate to intense staining divided by positively-staining cells (i.e., CYP1A ICC Index; $n = 3$ experiments $\pm$ S.E.M.). Enzyme activity for glass adherent and non-adherent cell populations was determined by an *ex vivo* EROD assay and data was expressed as (C) mean pmoles of resorufin /min /mg protein ($n = 4 - 8$ experiments $\pm$ S.E.M.). *,#,® Significantly different from the vehicle, 2, or 20 $\mu\text{g BaP/g}$ BW exposure groups within the designated cell population ($p \leq 0.05$), respectively.



BaP /g BW failed to demonstrate elevated CYP1A protein levels in adherent immune cells compared to the vehicle control. Considering the CYP1A-positive adherent and non-adherent cell populations, cells obtained from the highest BaP exposure group exhibited significantly more moderately- to intensely-staining cells compared to the vehicle control and the lower BaP treatment groups (Figure 18B).

6. CYP1A Enzyme Activity in Isolated Kidney Mononuclear Cells

CYP1A enzyme activity within isolated kidney mononuclear cells was significantly induced in non-adherent cells recovered from fish exposed to 200 µg BaP/g BW compared to the vehicle control and 2 µg BaP /g BW treatment group (Figure 18C). Although adherent cells possessed relatively higher EROD activity compared to non-adherent cells from all treatment groups, no significant BaP-induced changes in CYP1A enzyme activity were observed for this particular cell population.

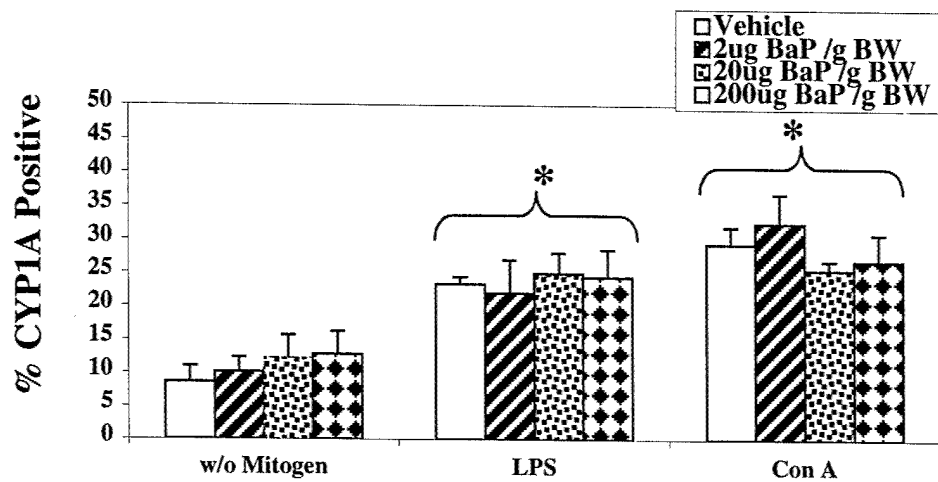
7. CYP1A Protein Expression in Mitogen-Stimulated Splenic

Lymphocytes

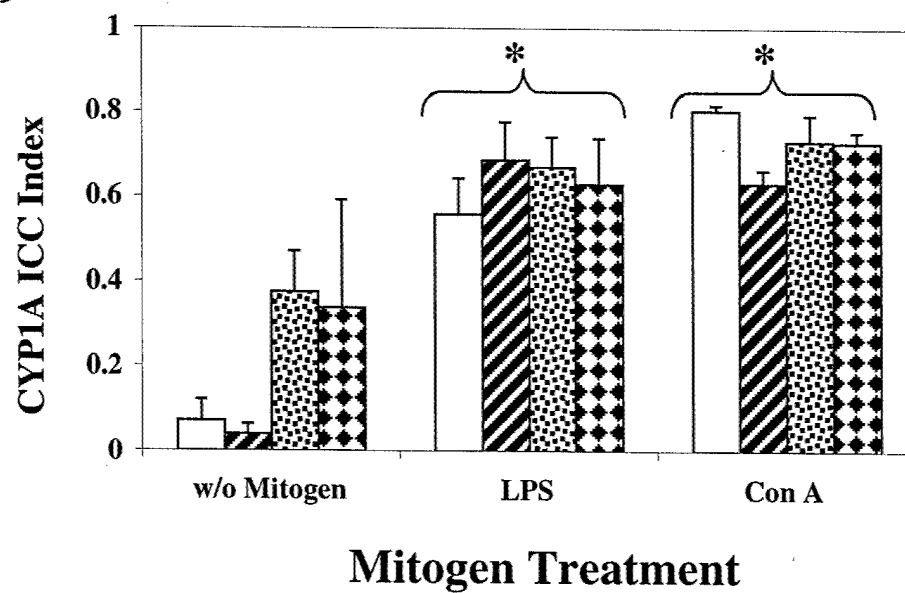
Exposure to BaP had no effect upon the percentage of CYP1A positive cells or CYP1A ICC Indices for unstimulated, LPS-, or Con A-stimulated splenocyte cultures. However, a significantly higher percentage of CYP1A positive cells (Figure 19A) and CYP1A ICC Indices (Figure 19B) were observed between LPS- or Con A-stimulated cells and unstimulated cultures, irrespective of BaP treatment. EROD activity was below detectable limits for all splenic cell cultures.

Figure 19. Analysis of CYP1A protein expression in mitogen-stimulated or unstimulated kidney mononuclear cells 48 h post-BaP injection. Medaka were injected IP with either vehicle or BaP at 2, 20, or 200 $\mu\text{g/g}$ BW prior to isolation of splenocytes. Expression of CYP1A protein was assessed within non-adherent splenocytes by CYP1A immunocytochemistry (ICC) 24 h following *in vitro* exposure to vehicle, LPS, or Con A. Data was expressed as either (A) mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; $n = 3$ experiments $\pm$ S.E.M.) or (B) mean relative frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index; $n = 3$ experiments $\pm$ S.E.M.). * Significantly different (irrespective of BaP treatment) from unstimulated cell cultures as analyzed by a 2-way ANOVA ($p \leq 0.05$).

A



B



C. SPECIFIC AIM 3

1. *In Vivo Studies*

a. Effects of Exposure to Benzo[e]pyrene (BeP) on Immune Function and CYP1A Expression /Activity

Plaque-forming cell numbers from fish exposed to BeP were similar to those observed in control fish (Figure 20). Exposure to BeP also failed to induce CYP1A protein levels or EROD activity in either hepatic or kidney microsomes (Figures 21 & 22).

Levels of CYP1A protein expressed within the liver, kidney, and spleen of fish injected with BeP were analyzed by IHC (Table 5) . Analysis of liver hepatocytes recovered from fish exposed to 400 µg BeP /g BW revealed a significantly higher CYP1A IHC Index compared to those injected with 2 µg BeP /g BW; no differences in renal tubular CYP1A expression were observed for any treatment group. A significantly higher CYP1A IHC index for kidney hematopoietic tissue was seen in the 400 µg BeP /g BW treatment group compared to the 200 µg BeP /g BW treatment group; however, kidney hematopoietic tissue from fish receiving 0 – 20 µg BeP /g BW doses demonstrated no staining. A significantly higher CYP1A IHC Index was observed in the splenic endothelia of fish receiving 400 µg BeP /g BW compared to the 20 and 200 µg BeP /g BW treatment groups.

Figure 20. Effects of IP exposure to benzo[e]pyrene (BeP) upon medaka PFC numbers. Medaka were injected IP with either vehicle or BeP at 2, 20, 200, or 400 $\mu\text{g/g}$ BW and immunized 48 h post-injection with SRBCs for later determination of kidney PFC numbers. Values represent the mean number of plaques / 2×10^6 kidney cells ($n = 3$ experiments $\pm$ S.E.M.).

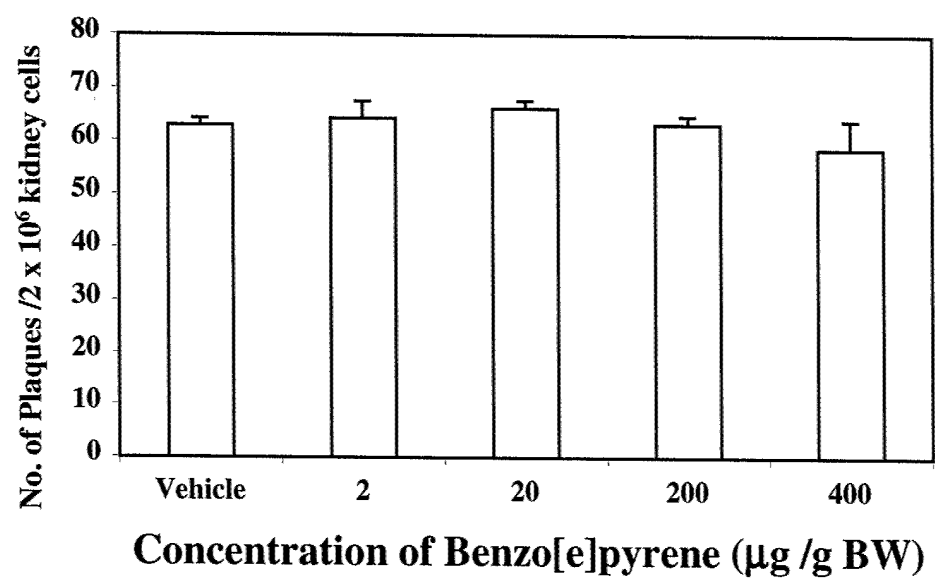


Figure 21. ELISA analysis of liver and kidney microsomal CYP1A protein levels 48 h post-BeP injection. Medaka were injected IP with either vehicle or BeP at 2, 20, 200, or 400 $\mu\text{g/g}$ BW prior to preparation of microsomes from individual livers (3 – 5 independent samples /treatment group /experiment) or pooled kidney samples (one pooled sample of 3 – 10 organs /treatment group /experiment). Relative levels of (A) hepatic and (B) kidney microsomal CYP1A protein as determined from ELISA using monoclonal C10-7 antibody as a probe. Values represent the mean absorbance at 405 nm ($n = 3$ independent experiments $\pm$ S.E.M.).

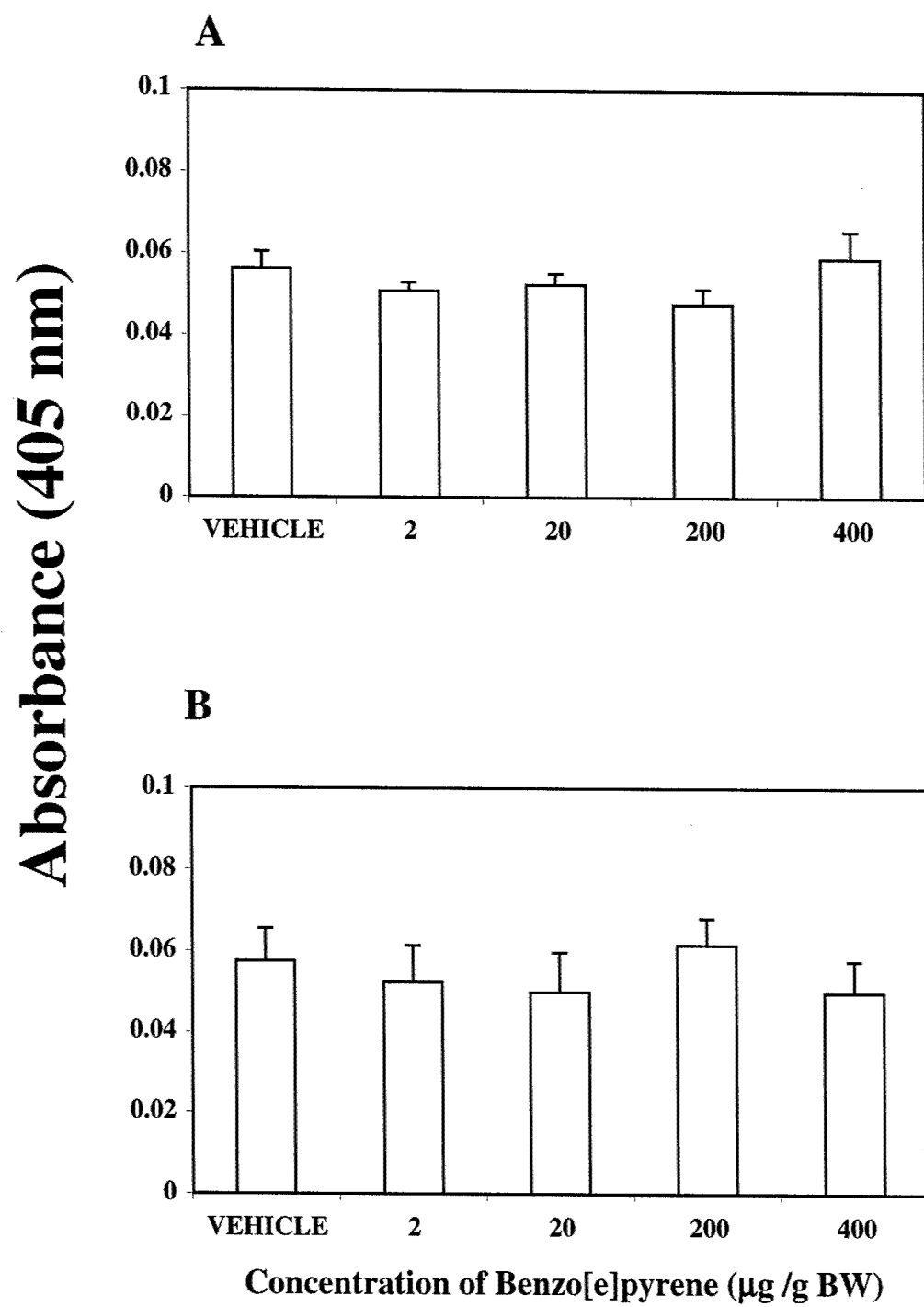
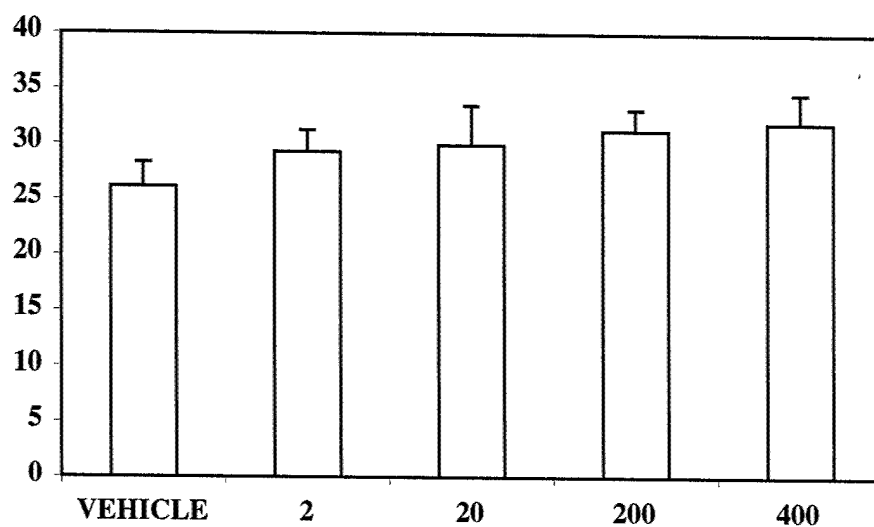


Figure 22. EROD activity of liver and kidney microsomes at 48 h post-BeP injection. Medaka were injected IP with either vehicle or BeP at 2, 20, 200, or 400 $\mu\text{g/g}$ BW prior to preparation of microsomes from individual livers (3 independent samples per treatment group per experiment) or pooled kidneys (one pooled sample of 3 – 10 organs per treatment group per experiment). Relative levels of (A) hepatic and (B) kidney microsomal EROD activity were determined using an EROD microtitre assay. Values represent the mean pmoles of resorufin /min /mg protein ($n = 3$ independent experiments $\pm$ S.E.M.).

pmoles resorufin /min /mg protein

A



B

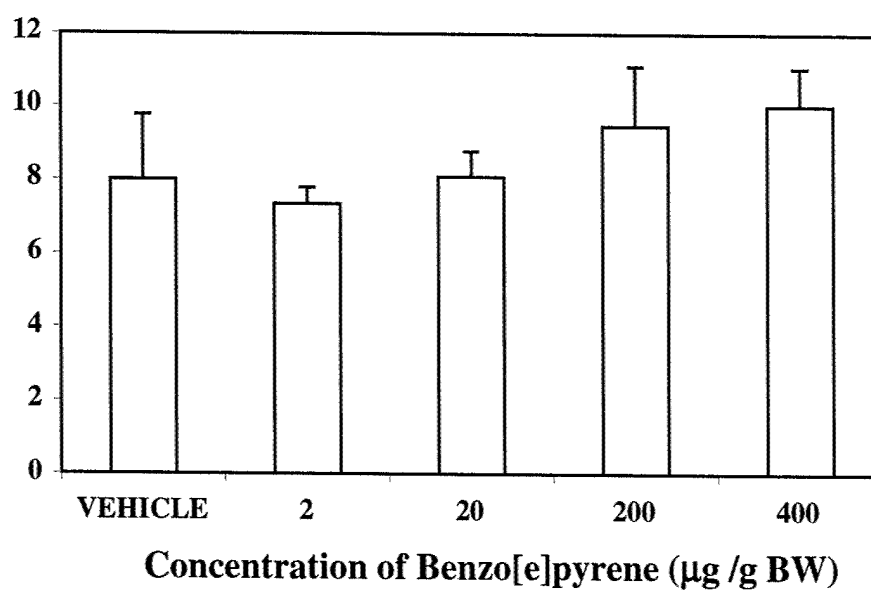


Table 5.

Effects of benzo[e]pyrene (BeP) on CYP1A immunohistochemistry (IHC) in medaka hepatocytes, kidney tubules and hematopoietic tissue, and splenic endothelia 48 h post-injection

BeP concentration ($\mu\text{g/g BW}$)	CYP1A IHC Index ^a			
	Liver	Kidney		Spleen
	Hepatocytes	Tubules	Hematopoietic Tissue	Endothelia
0	3.50 ± 0.67	4.00 ± 0.66	ND ^b	ND
2	$2.25 \pm 0.43^*$	3.67 ± 0.44	ND	ND
20	4.25 ± 0.66	4.92 ± 0.60	ND	$0.17 \pm 0.17^*$
200	4.33 ± 0.73	3.75 ± 0.43	$0.17 \pm 0.17^*$	$1.00 \pm 0.76^\#$
400	$5.50 \pm 0.25^*$	5.50 ± 1.00	$1.08 \pm 0.08^*$	$3.83 \pm 0.17^{*,\#}$

^aMean CYP1A IHC Index determined 48 h post-BeP injection ($n = 3$ individual experiments; 4 fish per treatment group $\pm$ S.E.M.).

^b ND = no detectable CYP1A staining (CYP1A IHC Index = 0).

^{*,#} Within a particular tissue, data with the same symbol are significantly different from each other ($p \leq 0.05$).

b. Effects of Concurrent Exposure to BaP and α -Naphthoflavone (ANF) on Immune Function and CYP1A Expression /Activity

While treatment with 200 μ g BaP /g BW significantly suppressed PFC numbers, BaP administration in combination with 200 μ g ANF /g BW completely ameliorated this effect (Figure 23).

Exposure to either 200 μ g BaP /g BW or BaP + ANF (200 μ g /g BW each) significantly induced both hepatic (Figure 24A) and kidney (Figure 24B) microsomal CYP1A protein. In addition, fish treated with either BaP or BaP + ANF had significantly increased liver microsomal CYP1A protein expression compared to those injected with ANF alone. ANF alone had no effect on either liver or kidney microsomal CYP1A protein expression.

Exposure of fish to 200 μ g BaP /g BW resulted in significant induction of hepatic (Figure 25A) and renal (Figure 25B) EROD activity compared to vehicle control. Treatment with either ANF alone or BaP + ANF had no effect on EROD activity. Induced CYP1A activity within liver and kidney microsomes from BaP-exposed fish were also significantly greater than those from ANF alone or BaP + ANF treatment groups.

Relative levels of CYP1A protein expressed within liver, kidney, and spleen following simultaneous exposure to BaP and ANF, as analyzed by IHC, are summarized in Table 6. Significantly greater CYP1A IHC Indices were observed for liver hepatocytes of BaP, ANF, and BaP + ANF treatment groups compared to vehicle

Figure 23. Effects of concurrent exposure to BaP and α -naphthoflavone (ANF) upon medaka PFC numbers. Medaka were injected IP with BaP alone (200 μ g /g BW), ANF alone (200 μ g /g BW), BaP + ANF in combination (200 μ g /g BW each), or vehicle. Forty-eight hours post-injection, medaka were immunized with SRBCs for later determination of PFC number. Values represent the mean number of plaques /2 x 10⁶ kidney cells (n = 3 experiments $\pm$ S.E.M.). * Significantly different from vehicle control, ANF alone, and ANF + BaP treatment groups ($p \leq 0.05$).

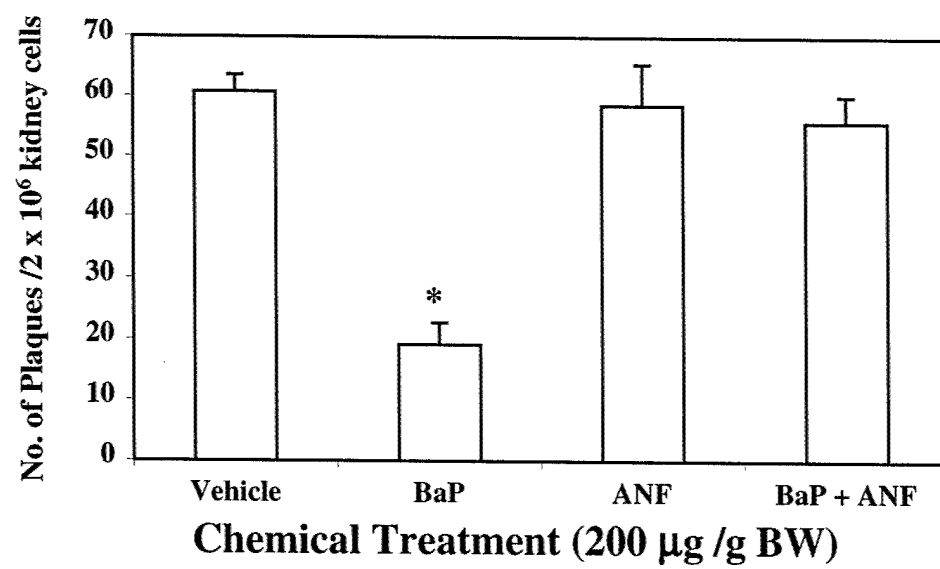


Figure 24. ELISA analysis of liver and kidney microsomal CYP1A protein levels 48 h following concurrent exposure to BaP and ANF. Medaka were injected IP with BaP alone (200 $\mu\text{g/g BW}$), ANF alone (200 $\mu\text{g/g BW}$), BaP + ANF in combination (200 $\mu\text{g/g BW}$ each), or vehicle prior to preparation of microsomes from individual livers (3 independent samples /treatment group /experiment) or pooled kidney samples (one pooled sample of 3 – 10 organs /treatment group /experiment). Relative levels of (A) hepatic and (B) kidney microsomal CYP1A protein were determined by ELISA using monoclonal C10-7 antibody as a probe. Values represent the mean absorbance at 405 nm ($n = 3$ independent experiments $\pm$ S.E.M.). * Significantly different from vehicle control ($p \leq 0.05$). *Significantly different from the ANF treatment group ($p \leq 0.05$).

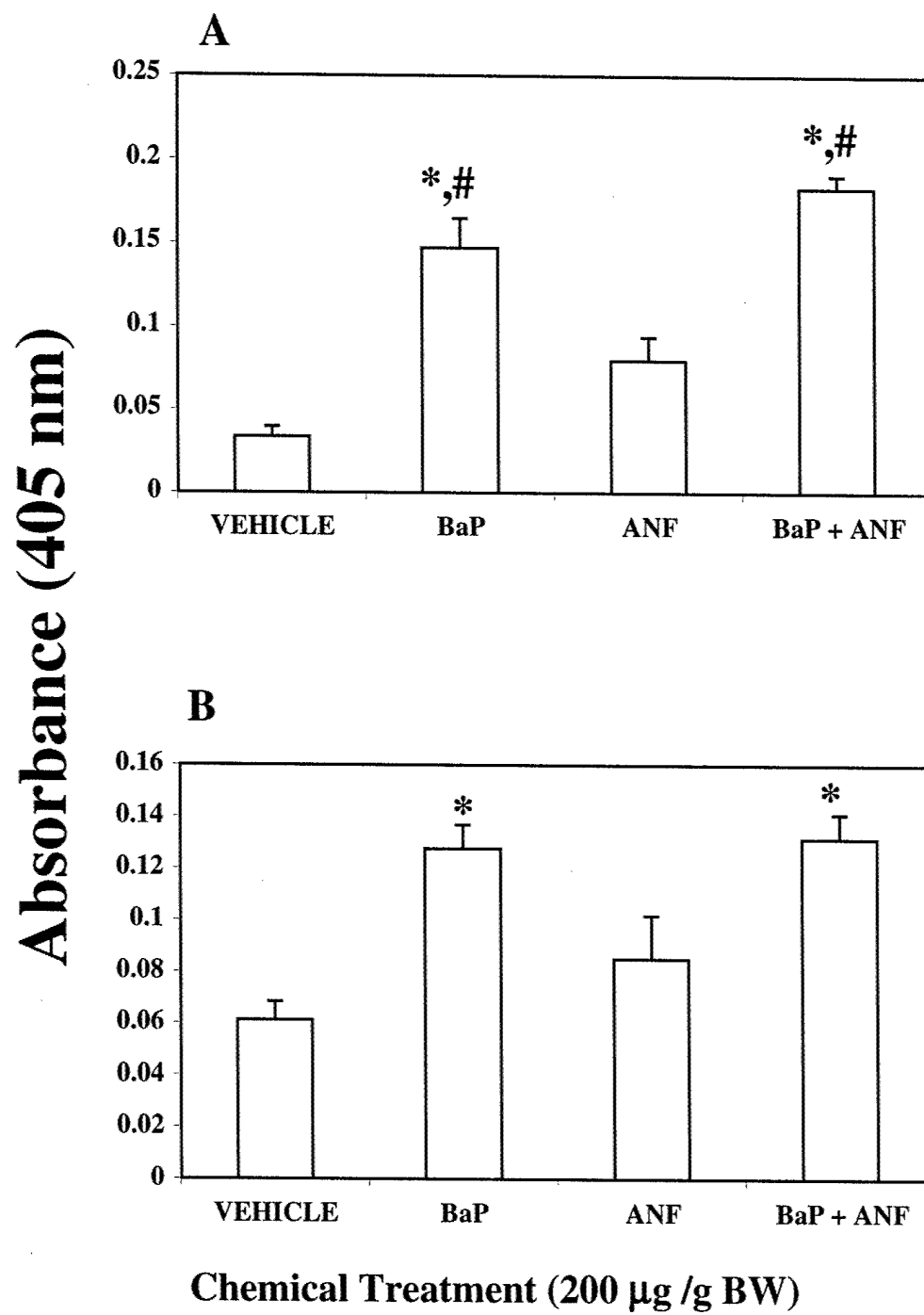
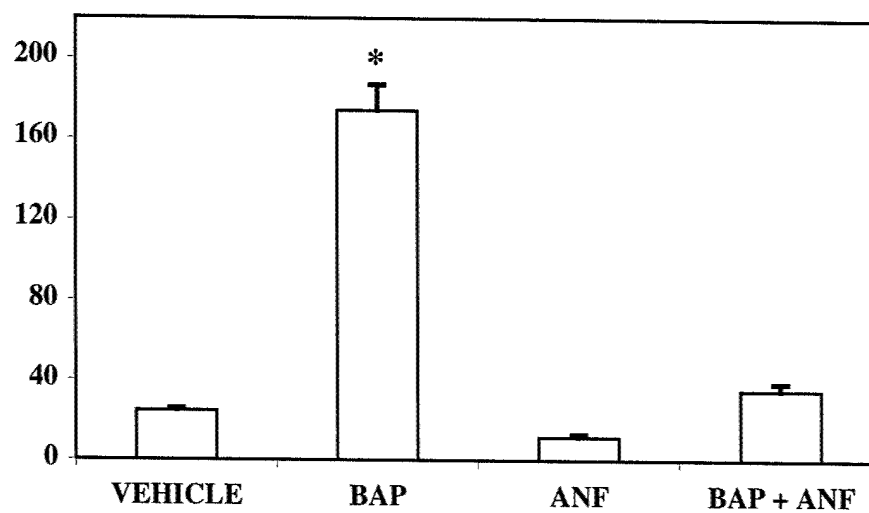


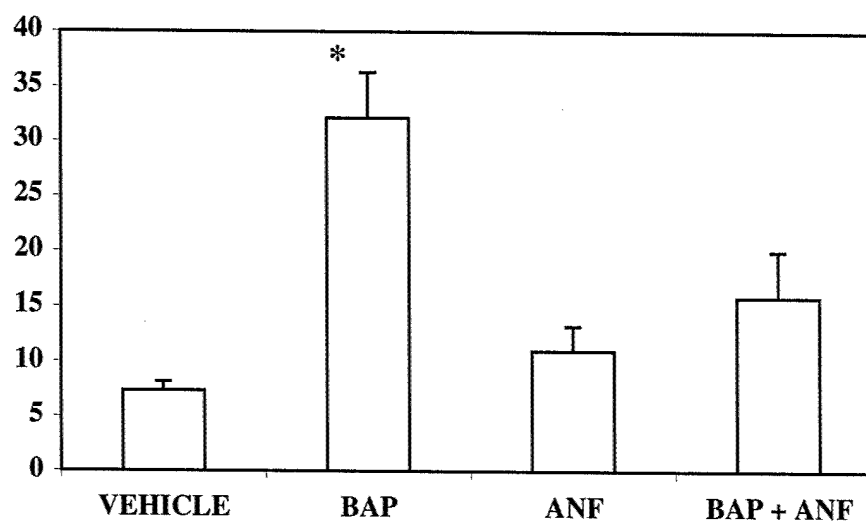
Figure 25. EROD activity of liver and kidney microsomes 48 h following concurrent exposure to BaP and ANF. Medaka were injected IP with BaP alone (200 µg /g BW), ANF alone (200 µg /g BW), BaP + ANF in combination (200 µg /g BW each), or vehicle control prior to preparation of microsomes from individual livers (3 - 5 independent samples /treatment group /experiment) or pooled kidney samples (one pooled sample of 3 - 10 organs /treatment group /experiment). Relative levels of (A) hepatic and (B) kidney microsomal EROD activity were determined using an EROD microtitre assay. Values represent the mean pmoles of resorufin /min /mg protein (n = 4 independent experiments ± S.E.M.). * Significantly different from vehicle control, ANF, and ANF + BaP treatment groups ($p \leq 0.05$).

pmoles resorufin /min /mg protein

A



B



Chemical Treatment (200 μ g /g BW)

Table 6.

Effects of benzo[a]pyrene (BaP) alone, α -naphthoflavone (ANF) alone, or BaP and ANF in combination on CYP1A immunohistochemistry (IHC) in medaka hepatocytes, kidney tubules and hematopoietic tissue, and splenic endothelia 48 h post-injection

Treatment ^a	CYP1A IHC Index ^b			
	Liver	Kidney		Spleen
	Hepatocytes	Tubules	Hematopoietic Tissue	Endothelia
Vehicle	3.75 $\pm$ 0.87 ^{*,#,@}	4.50 $\pm$ 1.50 ^{*,#,@}	ND ^c	ND
BaP	10.25 $\pm$ 0.50 ^{*,§}	8.75 $\pm$ 0.66 [*]	4.67 $\pm$ 0.73 [*]	7.42 $\pm$ 0.33 [*]
ANF	7.25 $\pm$ 0.25 ^{*,§,%}	8.25 $\pm$ 0.43 [#]	0.75 $\pm$ 0.38 [*]	3.33 $\pm$ 0.73 ^{*,#}
BaP + ANF	12.00 $\pm$ 0.87 ^{@,%}	9.25 $\pm$ 0.25 [@]	3.33 $\pm$ 0.83	7.25 $\pm$ 0.52 [#]

^aMedaka were injected IP with either BaP (200 μ g /g BW), ANF (200 μ g /g BW), BaP plus ANF (200 μ g of each compound /g BW), or the corn-oil vehicle. Forty-eight hr post-injection, liver, kidney, and spleen were excised for analysis of CYP1A IHC Index.

^bMean CYP1A IHC Index determined 48 h post- injection (n = 3 individual experiments; 4 fish per treatment group $\pm$ S.E.M.).

^c ND = no detectable CYP1A staining (CYP1A IHC Index = 0).

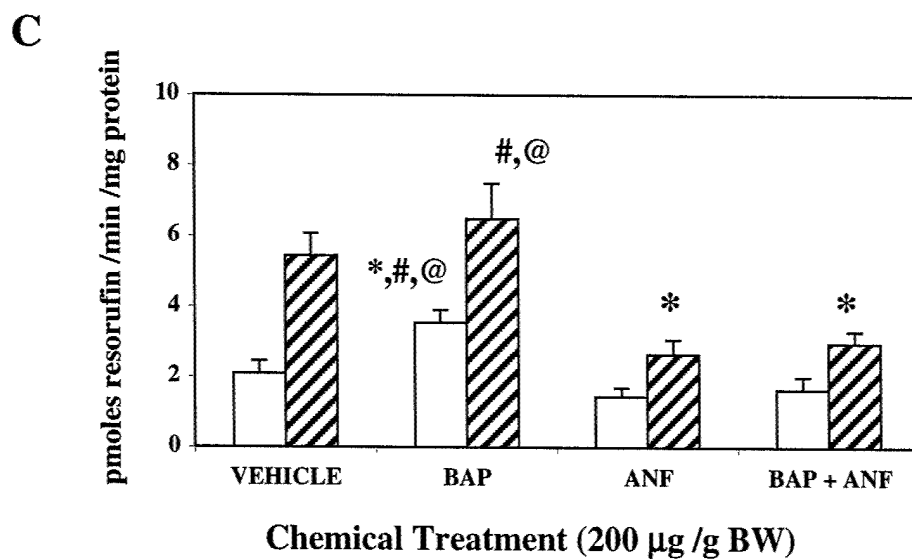
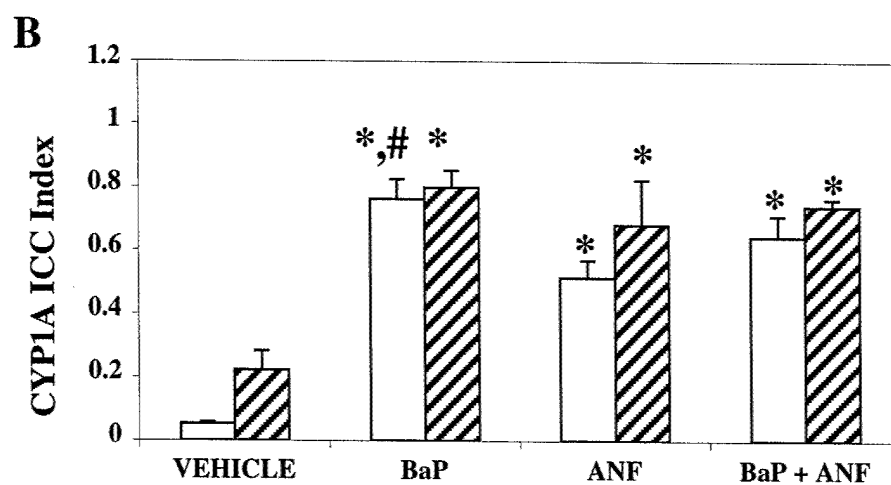
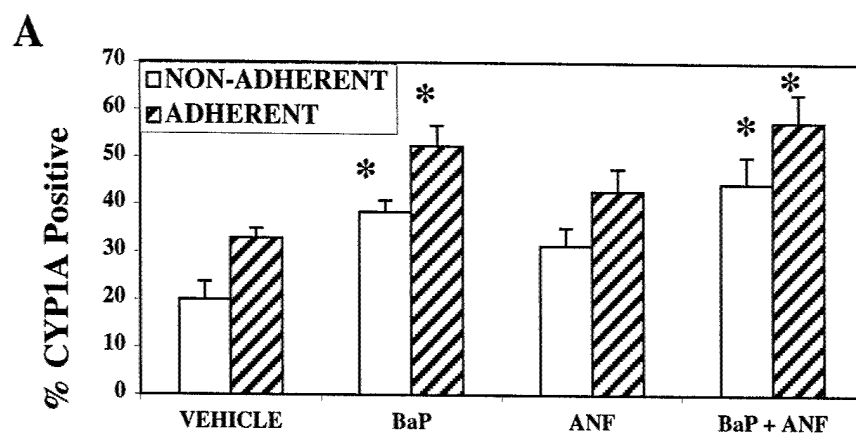
^{*,#,@,%,§} Within a particular tissue, data with the same symbol are significantly different from each other ($p \leq 0.05$).

control. In addition, hepatocyte staining was also significantly greater for the BaP and BaP + ANF treatment groups compared to cells from fish treated with ANF alone. Kidney tubules in fish from the BaP, ANF, and BaP + ANF treatment groups had significantly greater CYP1A IHC indices compared to vehicle controls. While vehicle control fish had undetectable levels of CYP1A expression in kidney hematopoietic tissue, exposure to BaP significantly increased CYP1A IHC Index (compared to the ANF alone treatment group); fish treated with BaP + ANF possessed only modestly higher levels of CYP1A expression. While CYP1A expression in the splenic endothelia of vehicle- and ANF-exposed fish was undetectable and CYP1A IHC Indices were significantly increased in the BaP- and BaP + ANF-exposed fish compared to ANF-exposed animals.

Immunocytochemical analysis of both glass adherent and non-adherent kidney mononuclear cells from fish exposed to either BaP alone or BaP + ANF revealed a significantly higher percentage of cells expressing CYP1A protein compared to vehicle control (Figure 26A). In addition, significantly more cells expressed moderate to high levels of CYP1A (i.e., CYP1A ICC Index) within CYP1A-positive adherent and non-adherent cell populations obtained from BaP-, ANF-, and BaP + ANF-exposed fish compared to vehicle control (Figure 26B). A significantly higher CYP1A ICC Index was also calculated for the non-adherent cell population from BaP-treated animals (compared to those from the ANF alone treatment group).

EROD activity was significantly elevated in non-adherent cells from BaP-exposed fish compared to those from vehicle control, ANF, and BaP + ANF treatment

Figure 26. Analysis of CYP1A protein expression and activity in isolated kidney mononuclear cells 48 h following concurrent exposure to BaP and ANF. Medaka were injected IP with BaP alone (200 μg /g BW), ANF alone (200 μg /g BW), BaP + ANF in combination (200 μg /g BW each), or vehicle control prior to isolation of kidney mononuclear cells. Expression of CYP1A protein was assessed for both glass adherent and non-adherent mononuclear cells using CYP1A immunocytochemistry (ICC). Data was expressed as either (A) the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; $n = 3$ experiments $\pm$ S.E.M.) or (B) the mean relative frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index; $n = 3$ experiments $\pm$ S.E.M.). Activity of CYP1A protein within glass adherent and non-adherent cell populations was determined by an *ex vivo* EROD assay; data was expressed as (C) the mean pmol of resorufin /min /mg protein ($n = 3$ experiments $\pm$ S.E.M.). *,#,@ Significantly different from the vehicle, ANF only, or ANF + BaP exposure groups within the designated cell population ($p \leq 0.05$), respectively.



groups (Figure 26C). Glass adherent cells from ANF- and BaP + ANF-exposed fish had significantly lower EROD activity compared to either the vehicle control or BaP-treated groups. The EROD activity of adherent mononuclear cells from fish treated with BaP alone was not significantly different from that of the vehicle control.

*c. Effects of Concurrent Exposure to BaP and
Dehydroepiandrosterone (DHEA) on Immune Function and CYP1A Expression
/Activity*

While treatment of fish with 200 µg BaP /g BW significantly suppressed PFC numbers (compared to vehicle control), levels reached vehicle control values when BaP was administered in combination with 200 µg DHEA /g BW (Figure 27).

Levels of liver (Figure 28A) and kidney (Figure 28B) microsomal CYP1A protein were significantly greater in fish exposed to the BaP alone or BaP + DHEA compared to either the vehicle control or DHEA treatment group. Expression of CYP1A protein in liver and kidney from the DHEA treatment group was not significantly different from the vehicle control.

While exposure of fish to 200 µg BaP /g BW significantly induced hepatic (Figure 29A) and renal (Figure 29B) EROD activity (compared to vehicle control), treatment of fish with either DHEA alone or BaP + DHEA had no effect upon EROD activity. CYP1A activity within liver and kidney microsomes from BaP-exposed fish was also significantly greater than that measured in fish treated with DHEA alone.

Figure 27. Effects of concurrent exposure to BaP and dehydroepiandrosterone (DHEA) upon medaka PFC numbers. Medaka were injected IP with either vehicle control, BaP alone (200 µg /g BW), DHEA alone (200 µg /g BW), or BaP + DHEA in combination (200 µg /g BW each). Forty-eight hours post-injection medaka were immunized with SRBCs for later determination of PFC number. Values represent the mean number of plaques /2 x 10⁶ kidney cells (n = 3 experiments ± S.E.M.).

*Significantly different from vehicle control, DHEA alone, and DHEA + BaP treatment groups ($p \leq 0.05$).

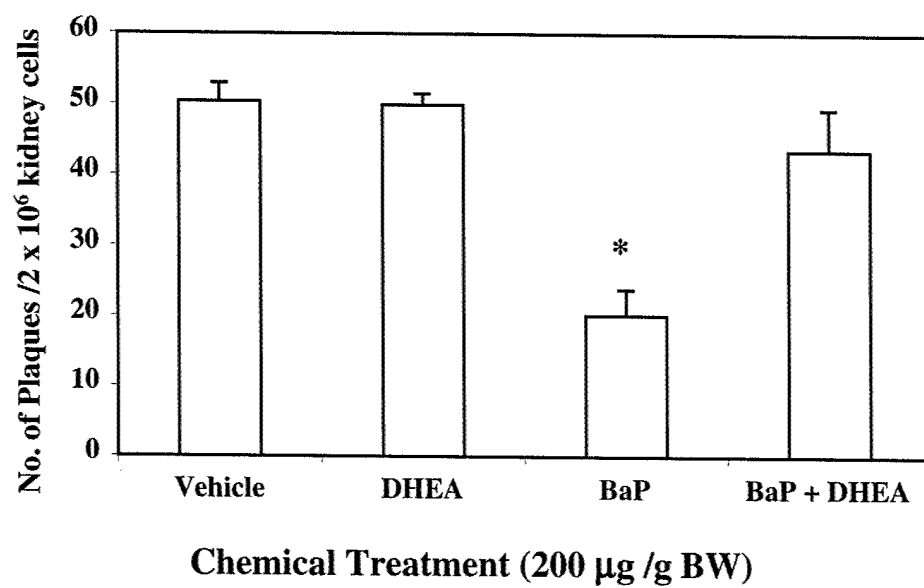


Figure 28. ELISA analysis of liver and kidney microsomal CYP1A protein levels 48 h following concurrent exposure of fish to BaP and DHEA. Medaka were injected IP with either BaP alone (200 µg /g BW), DHEA alone (200 µg /g BW), BaP + DHEA in combination (200 µg /g BW each), or vehicle control prior to preparation of microsomes from individual livers (3 independent samples /treatment group /experiment) or pooled kidney samples (one pooled sample of 3 – 10 organs /treatment group /experiment). Relative levels of (A) hepatic and (B) kidney microsomal CYP1A protein were determined by ELISA using monoclonal C10-7 antibody as a probe. Values represent the mean absorbance at 405 nm (n = 3 independent experiments ± S.E.M.). * Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the DHEA treatment group ($p \leq 0.05$).

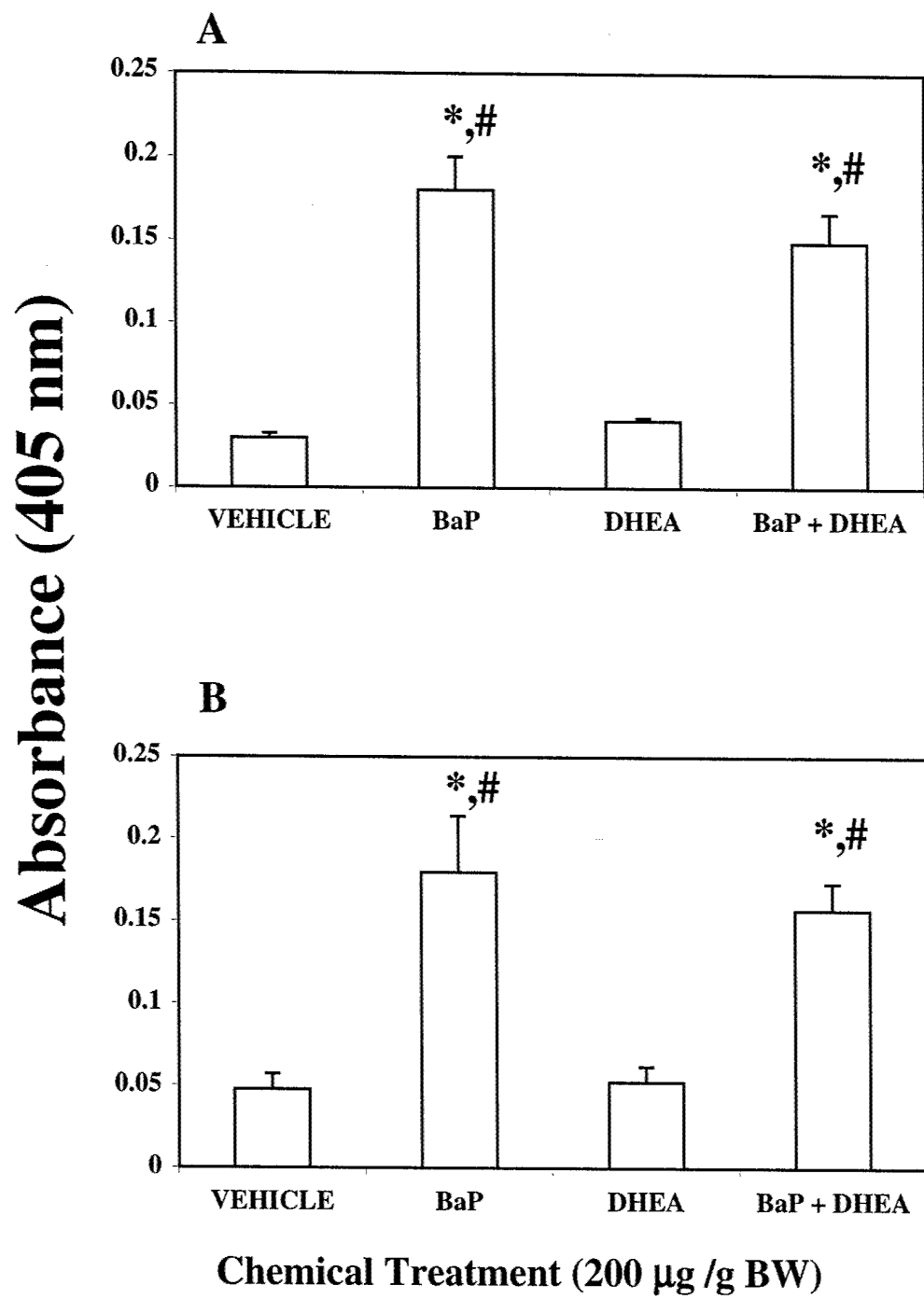
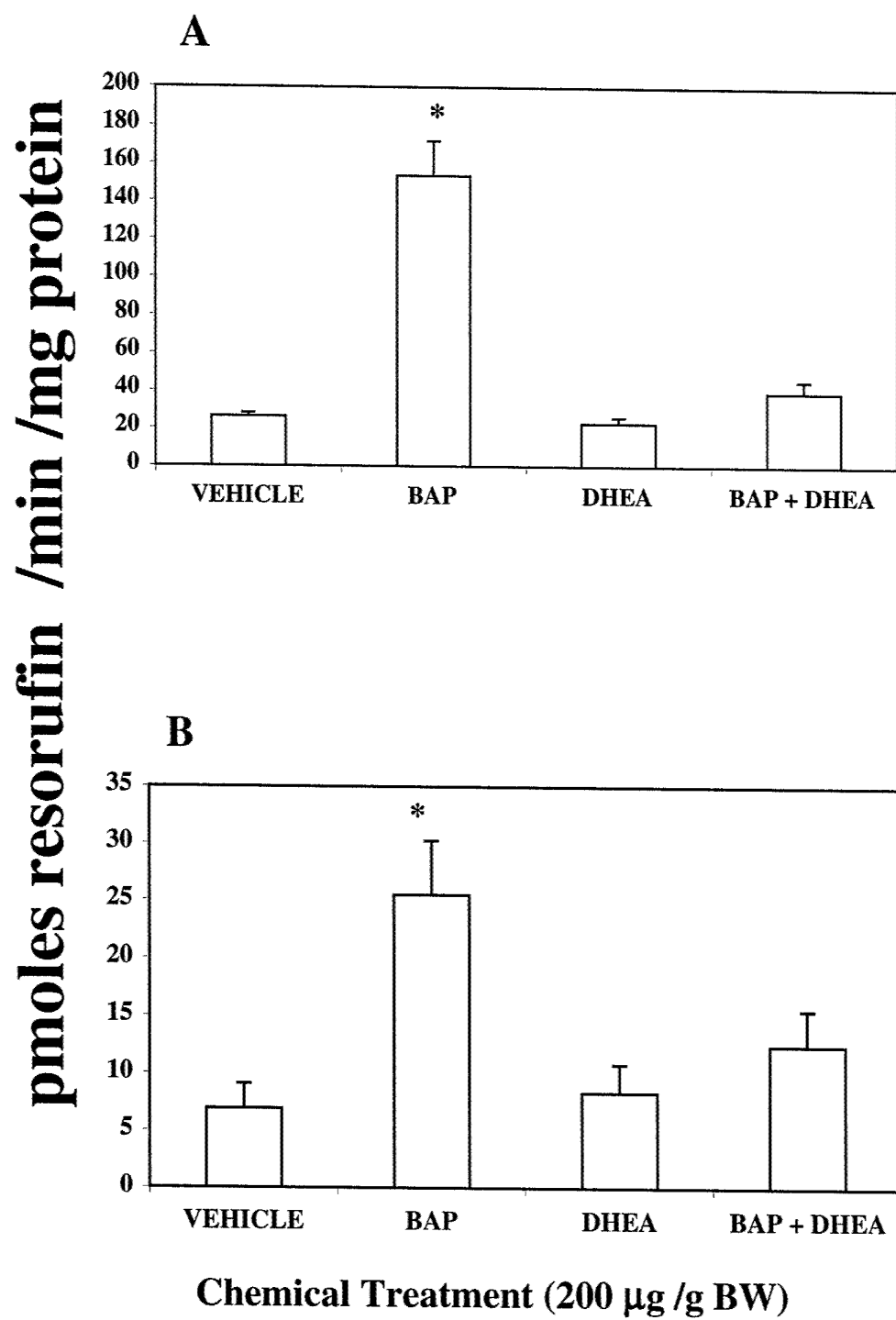


Figure 29. EROD activity of liver and kidney microsomes 48 h following concurrent exposure of fish to BaP and DHEA. Medaka were injected IP with BaP alone (200 μ g /g BW), DHEA alone (200 μ g /g BW), BaP + DHEA in combination (200 μ g /g BW each), or vehicle prior to preparation of microsomes from individual livers (3 - 5 independent samples /treatment group /experiment) or pooled kidney samples (one pooled sample of 3 - 10 organs /treatment group /experiment). Relative levels of (A) hepatic and (B) kidney microsomal EROD activity were determined by an EROD microtitre assay. Values represent the mean pmoles of resorufin /min /mg protein (n = 4 independent experiments $\pm$ S.E.M.). *Significantly different from vehicle control, DHEA alone, and DHEA + BaP treatment groups ($p \leq 0.05$).



Relative levels of CYP1A protein expressed within the liver, kidney, and spleen of BaP + DHEA-exposed fish are shown in Table 7. Liver hepatocytes and renal tubules recovered from BaP- and BaP + DHEA-treated fish expressed significantly greater levels of CYP1A than those treated with either vehicle or DHEA. Although neither the vehicle control- nor DHEA-treated fish demonstrated detectable levels of CYP1A expression in either kidney hematopoietic tissue or spleen endothelia, CYP1A protein was detected in these tissue types from BaP- and BaP + DHEA-exposed fish.

A significantly higher percentage of isolated kidney mononuclear cells possessing CYP1A protein was observed for both glass adherent and non-adherent cell populations from BaP- and BaP + DHEA-treated fish (compared to either the vehicle control or DHEA exposure group; Figure 30A). In addition, a significant increase in moderately- to intensely-staining cells was observed in the CYP1A-positive non-adherent cell populations obtained from either BaP or BaP + DHEA treatment groups compared to that in either the vehicle control or DHEA treatment groups (Figure 30B). Glass adherent mononuclear cells from BaP- and BaP + DHEA-exposed fish demonstrated only modestly higher CYP1A ICC Indices compared to that observed in cells from the vehicle control or DHEA exposure groups.

Non-adherent cells from BaP-exposed fish demonstrated significantly higher EROD activity compared to that seen in cells collected from either the vehicle control or DHEA treatment groups (Figure 30C). EROD activities of adherent mononuclear cells were not altered by any chemical treatments.

Table 7.

Effects of benzo[a]pyrene (BaP) alone, dehydroepiandrosterone (DHEA) alone, or BaP and DHEA in combination on CYP1A immunohistochemistry (IHC) in medaka hepatocytes, kidney tubules and hematopoietic tissue, and splenic endothelia 48 h post-injection

Treatment ^a	CYP1A IHC Index ^b			
	Liver	Kidney		Spleen
	Hepatocytes	Tubules	Hematopoietic Tissue	Endothelia
Vehicle	5.75 ± 0.66 ^{*,#}	4.50 ± 0.87 ^{*,#}	ND ^c	ND
BaP	9.25 ± 1.32 ^{*,@}	10.25 ± 1.00 ^{*,@}	4.00 ± 0.52	6.67 ± 0.67
DHEA	4.17 ± 0.96 ^{*,\$}	4.75 ± 0.90 ^{*,\$}	ND	ND
BaP + DHEA	9.5 ± 0.25 ^{*,\$}	11.67 ± 1.08 ^{*,\$}	2.83 ± 0.79	7.25 ± 0.63

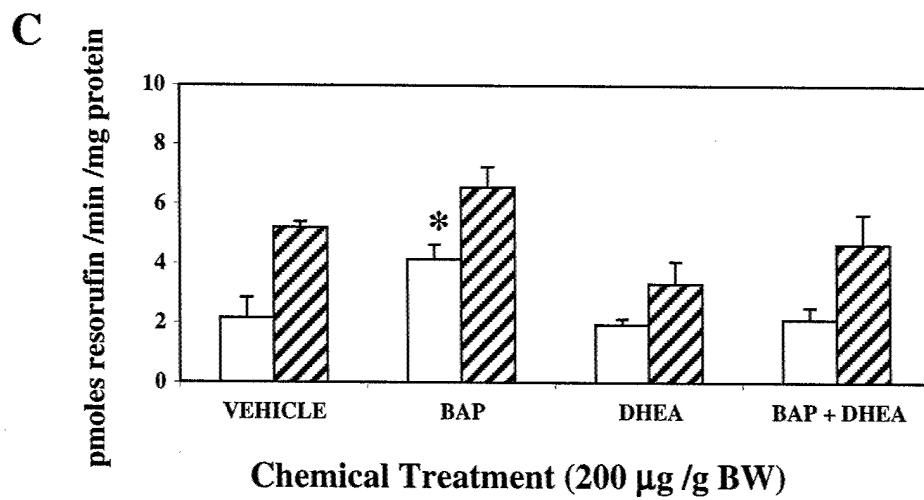
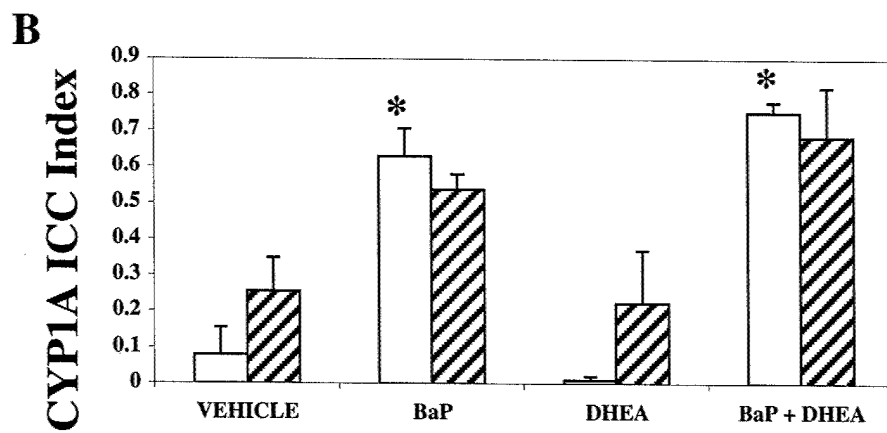
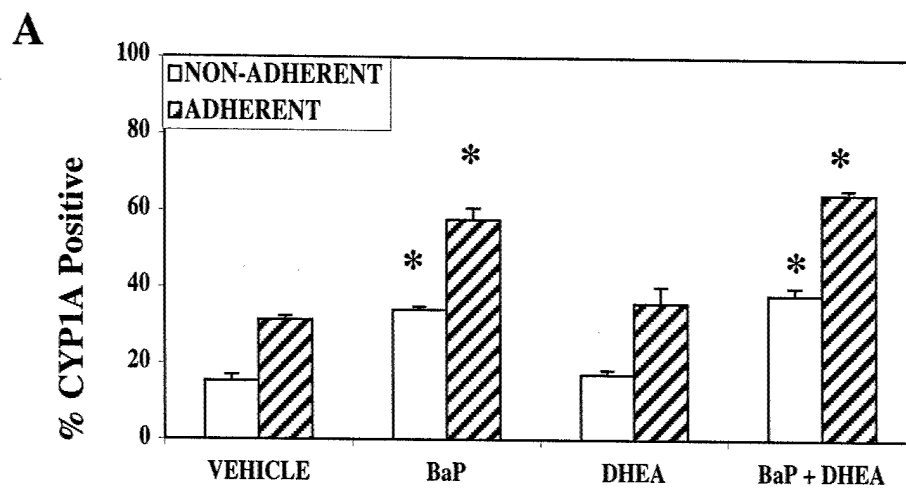
^aMedaka were injected IP with either BaP (200 µg /g BW), DHEA (200 µg /g BW), BaP + DHEA (200 µg of each compound /g BW), or the corn-oil vehicle. Forty-eight hr post-injection, liver, kidney, and spleen were removed for analysis of CYP1A IHC Index.

^bMean CYP1A IHC Index determined 48 h post- injection (n = 3 individual experiments; 4 fish per treatment group ± S.E.M.).

^cND = no detectable CYP1A staining (CYP1A IHC Index = 0).

^{*,#,@,%, \$} Within a particular tissue, data with the same symbol are significantly different from each other (p ≤ 0.05).

Figure 30. Analysis of CYP1A protein expression and activity in isolated kidney mononuclear cells 48 h following concurrent exposure of fish to BaP and DHEA. Medaka were injected IP with BaP alone (200 µg /g BW), DHEA alone (200 µg /g BW), BaP + DHEA in combination (200 µg /g BW each), or vehicle prior to isolation of kidney mononuclear cells. Expression of CYP1A protein was assessed within glass adherent and non-adherent mononuclear cells by CYP1A immunocytochemistry (ICC). Data was expressed as either (A) mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; n = 3 experiments ± S.E.M.) or (B) mean frequency of cells expressing moderate to intense staining ÷ by the number of positively-staining cells (i.e., CYP1A ICC Index; n = 3 experiments ± S.E.M.). CYP1A protein activity within glass adherent and non-adherent cell populations was determined by an *ex vivo* EROD assay and data was expressed as (C) the mean pmoles of resorufin /min /mg protein (n = 3 experiments ± S.E.M.). *Significantly different from vehicle control and the DHEA exposure groups within the indicated cell population ($p \leq 0.05$).



2. *In Vitro* Studies

a. *Effects of In Vitro BaP Exposure on Immune Function and CYP1A Expression /Activity*

Significantly lower PFC numbers were observed in cell cultures exposed to 1.0 μM BaP compared to both vehicle control and the lowest BaP treatment group (Figure 31); incubation with 0.01 μM BaP significantly decreased PFC numbers compared to the lowest BaP treatment group, but not compared to the vehicle control.

Following exposure to either 0.01 or 1.0 μM BaP, adherent and non-adherent cells expressed significantly greater levels of CYP1A protein after 24 h than did the vehicle control (Figure 32A). Non-adherent cells exposed to the highest BaP dose produced significantly greater numbers of CYP1A-positive cells than those cells treated with the two lower BaP concentrations. Cells treated with 0.01 μM BaP had a significantly higher percentage of CYP1A-positive non-adherent cells compared to cells treated with the lowest BaP dose. Significantly higher CYP1A ICC Indices were exhibited by adherent cells receiving either 0.01 or 1.0 μM BaP compared to the vehicle control (Figure 32B). CYP1A ICC Indices of adherent cells exposed to the highest BaP concentration were also significantly greater compared to the lowest BaP treatment group; non-adherent cells from the highest BaP treatment group had an CYP1A ICC Index only modestly higher than vehicle control.

Both adherent and non-adherent cells exposed *in vitro* to either 0.01 or 1.0 μM BaP demonstrated significantly higher EROD activity compared to vehicle control (Figure 32C); adherent cells treated with the lowest BaP concentration also exhibited a

Figure 31. Effects of *in vitro* exposure to BaP upon medaka PFC numbers. Isolated kidney mononuclear cells were exposed *in vitro* to either vehicle or BaP at 0.0001, 0.01, 1.0 μ M and subsequently to SRBCs. Nine days post-culture initiation, PFCs were enumerated and data was expressed as the mean number of specific PFCs / 1×10^6 cells ($n = 4$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the 0.0001 μ M BaP exposure group ($p \leq 0.05$).

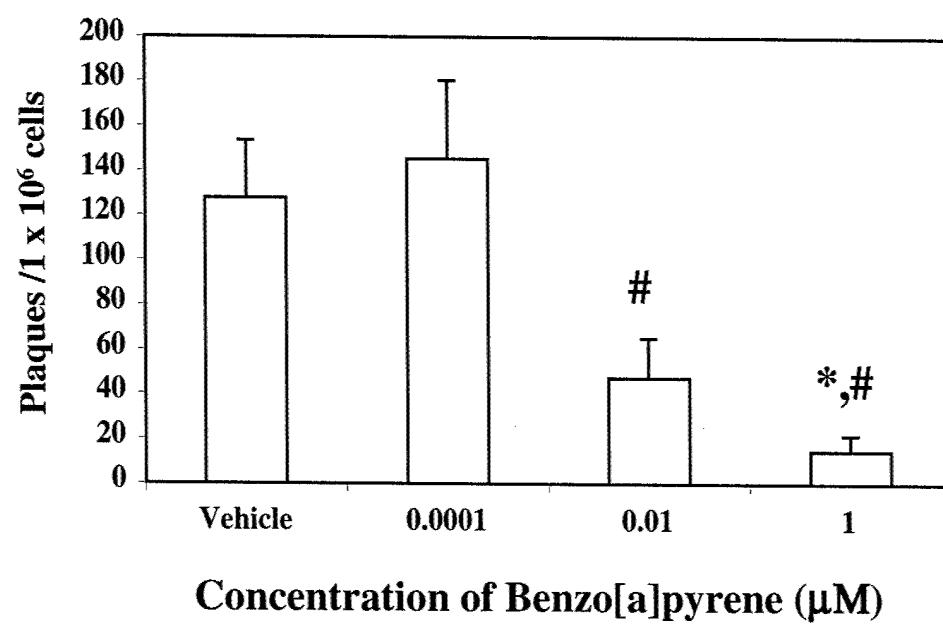
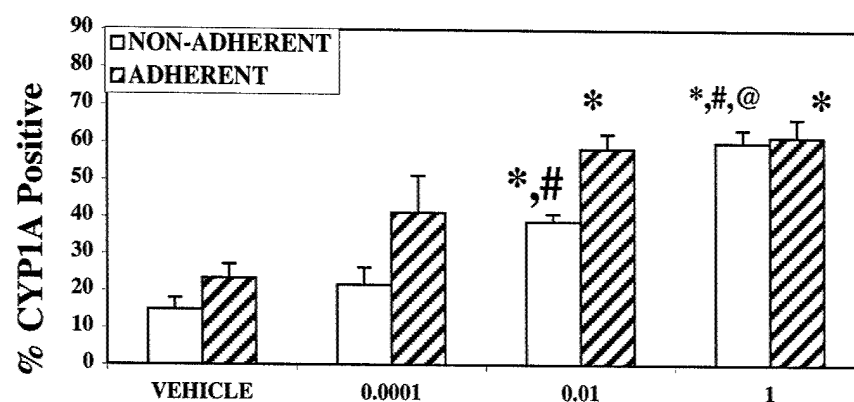
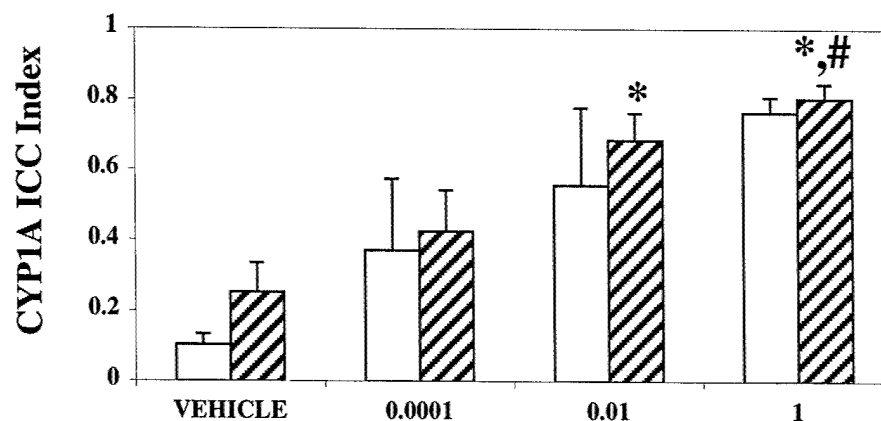


Figure 32. Analysis of CYP1A protein expression and activity in isolated kidney mononuclear cells 24 h following *in vitro* BaP exposure. Isolated kidney mononuclear cells were exposed *in vitro* to either vehicle or BaP at 0.0001, 0.01, 1.0 μ M. Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells by CYP1A immunocytochemistry (ICC) 24 h post-culture initiation. Data was expressed as either (A) the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; $n = 3$ experiments $\pm$ S.E.M.) or (B) the mean frequency of cells expressing moderate to intense staining $\div$ by positively-staining cells (i.e., CYP1A ICC Index; $n = 3$ experiments $\pm$ S.E.M.). CYP1A activity, as measured by an EROD assay, within adherent and non-adherent cell populations was determined; data was expressed as (C) the mean pmoles of resorufin /min /mg protein ($n = 3$ experiments $\pm$ S.E.M.). *,#,@ Significantly different from the vehicle, 0.0001, or 0.01 μ M BaP exposure groups within the designated cell population ($p \leq 0.05$), respectively.

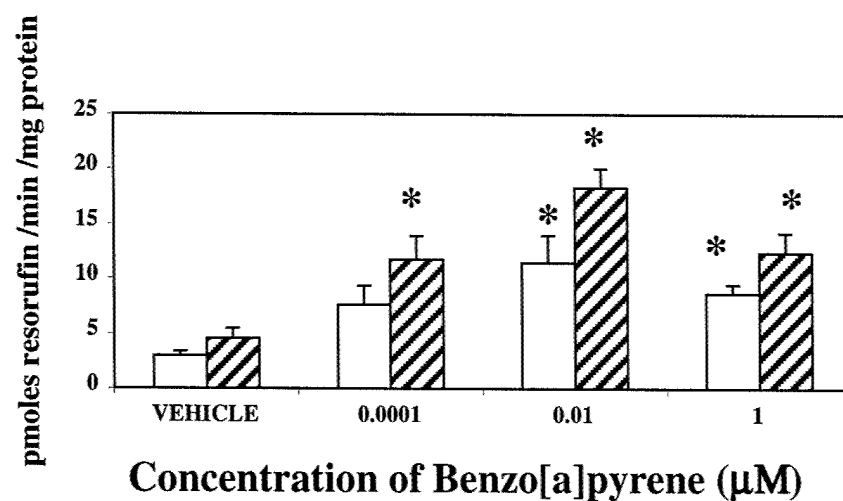
A



B



C



significantly higher EROD activity compared to vehicle control. Interestingly, both adherent and non-adherent cell populations exposed to 0.01 μM BaP had the highest mean EROD activity of any treatment group; though the effects were significant only versus the vehicle control.

b. Effects of In Vitro Exposure to BaP-Diol (BD) on Immune Function and CYP1A Expression /Activity

Exposure of cells to 1.0 μM BD significantly reduced PFC numbers compared to those in the vehicle control or the lower BD exposure groups (Figure 33). Treatment with either 0.0001 or 0.01 μM BD had no effect upon PFC number.

A significant increase in the percentage of mononuclear cells expressing CYP1A at 24 h post-culture initiation (compared to vehicle control and the lowest BD treatment group) was seen in non-adherent cells treated with the highest BD dose (Figure 34A). No differences in CYP1A ICC Index were observed between any of the treatment groups for either the adherent or non-adherent cell populations (Figure 34B). Exposure of non-adherent cells to 1.0 μM BD had significantly greater EROD activity compared to cells exposed to the vehicle (Figure 34C); adherent cells treated with 1.0 μM BD demonstrated only a slight induction of EROD activity.

Figure 33. Effects of *in vitro* exposure to BaP-Diol (BD) upon medaka mononuclear PFC numbers. Isolated kidney mononuclear cells were exposed *in vitro* to either vehicle or BD at 0.0001, 0.01, 1.0 μ M and subsequently immunized with SRBCs. Nine days post-culture initiation, PFCs were enumerated and data was expressed as the mean number of specific PFCs / 1×10^6 cells (n = 3 experiments $\pm$ S.E.M.).

*Significantly different from vehicle control, 0.0001, and 0.01 μ M BD exposure groups ($p \leq 0.05$).

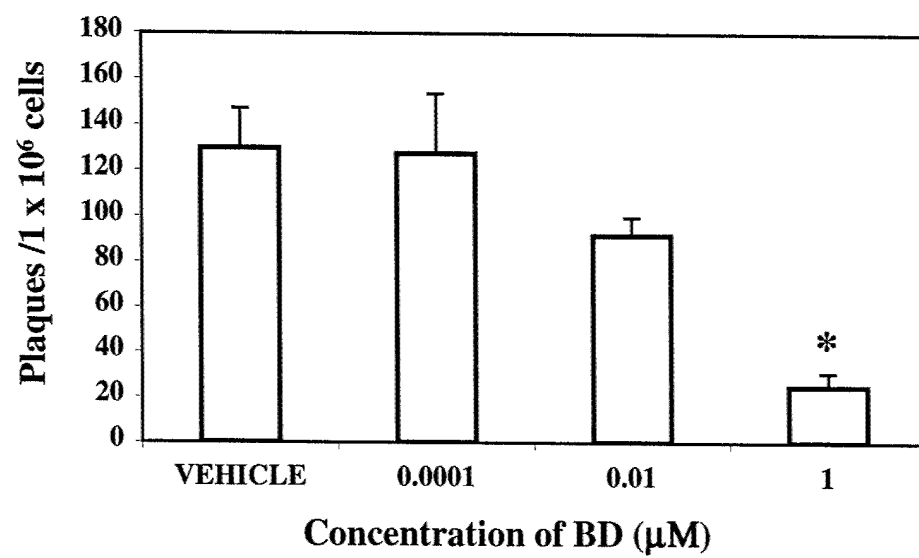
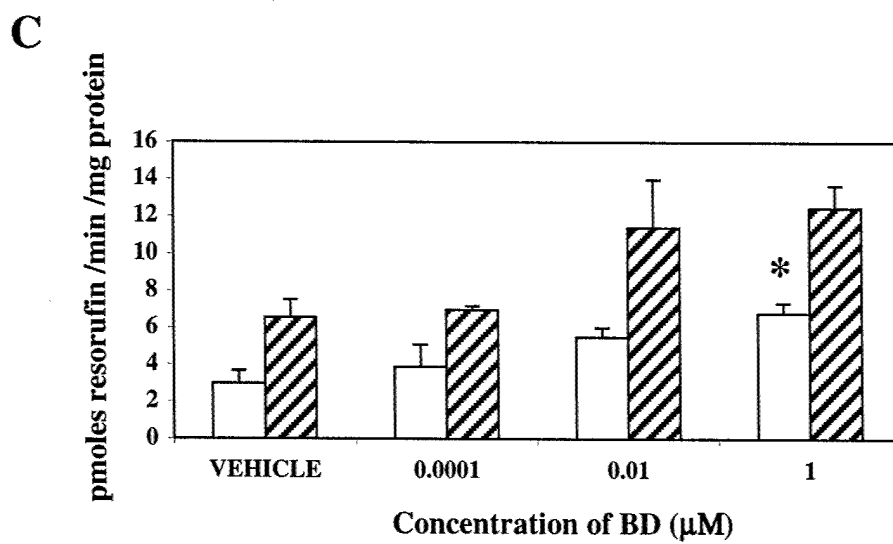
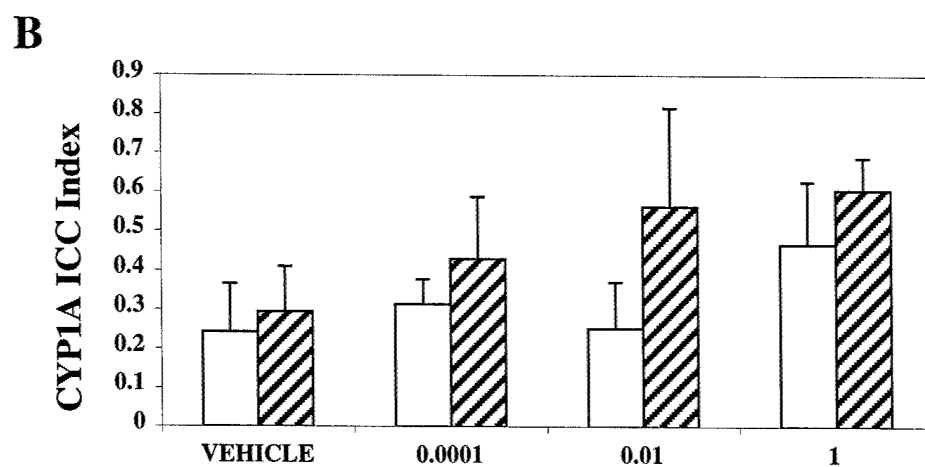
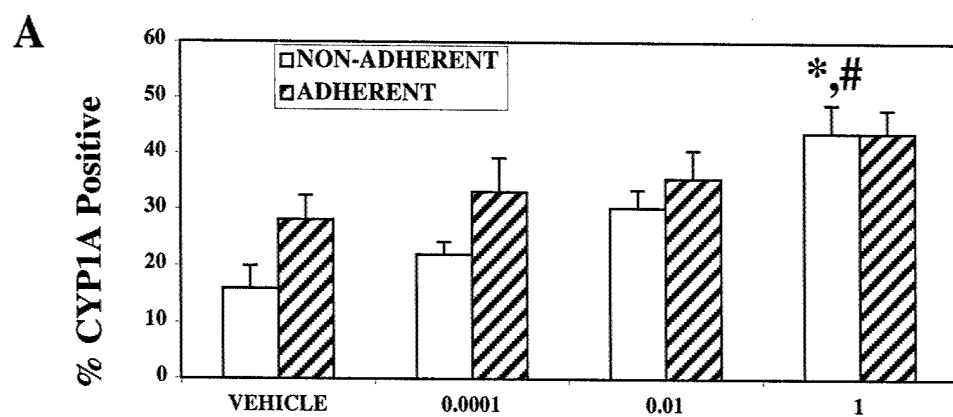


Figure 34. Analysis of CYP1A protein expression and activity in isolated kidney mononuclear cells 24 h following *in vitro* exposure to BD. Isolated kidney mononuclear cells were exposed *in vitro* to either vehicle or BD at 0.0001, 0.01, 1.0 μ M. Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells by CYP1A immunocytochemistry (ICC) 24 post-culture initiation. Data was expressed as either (A) the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; n = 3 experiments $\pm$ S.E.M.) or (B) the mean frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index; n = 3 experiments $\pm$ S.E.M.). Activity of CYP1A within adherent and non-adherent cell populations was determined *in vitro* by using an EROD microtitre assay; data was expressed as (C) the mean pmoles of resorufin /min /mg protein (n = 3 experiments $\pm$ S.E.M.). *# Significantly different from the vehicle or 0.0001 μ M BD exposure group within the designated cell population ($p \leq 0.05$), respectively.



c. Effects of In Vitro Exposure to BaP-Diolepoxide (BPDE) on Immune Function and CYP1A Expression /Activity

Significantly fewer PFCs were observed following exposure of cells to 0.01 and 1.0 μ M BPDE compared to those treated with vehicle control or the lowest BPDE exposure concentration (Figure 35); exposure to 0.0001 μ M BPDE had no effect upon PFC number. In addition, exposure of cells to BPDE at any concentration had no effect upon the number of cells expressing CYP1A protein or the CYP1A ICC Index for both adherent and non-adherent cell populations (Figure 36A & B). However, exposure to BPDE concentrations of 0.01 and 1.0 μ M significantly reduced EROD activities compared to that produced by the lowest BPDE concentration (Figure 36C); 1.0 μ M BPDE also significantly decreased EROD activity compared to the vehicle control.

d. Effects of Concurrent In Vitro Exposure to BaP /BaP Metabolites and 3'-Methoxy-4'-Nitroflavone (MNF) on Immune Function and CYP1A Expression /Activity

Exposure of cells to either 1.0 μ M BaP or concurrent exposure to 1.0 μ M BaP and 0.05 μ M MNF resulted in significantly reduced PFC numbers compared to vehicle control (Figure 37A); exposure to 0.05 μ M MNF alone slightly reduced PFC numbers (compared to the vehicle control). Exposure of cells to either 1.0 μ M BD or concurrent exposure to 1.0 μ M BD and 0.05 μ M MNF (Figure 37B) reduced PFC numbers compared to the vehicle control and MNF treatment groups. In addition, exposure to

Figure 35. Effects of *in vitro* exposure to BaP-Diolepoxide (BPDE) upon medaka mononuclear PFC numbers. Isolated kidney mononuclear cells were exposed *in vitro* to either vehicle control or BPDE at 0.0001, 0.01, 1.0 μ M and subsequently immunized with SRBCs. Nine days post-culture initiation, PFCs were enumerated and data was expressed as the mean number of specific PFCs / 1×10^6 cells ($n = 3$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control and 0.0001 μ M BPDE exposure groups ($p \leq 0.05$).

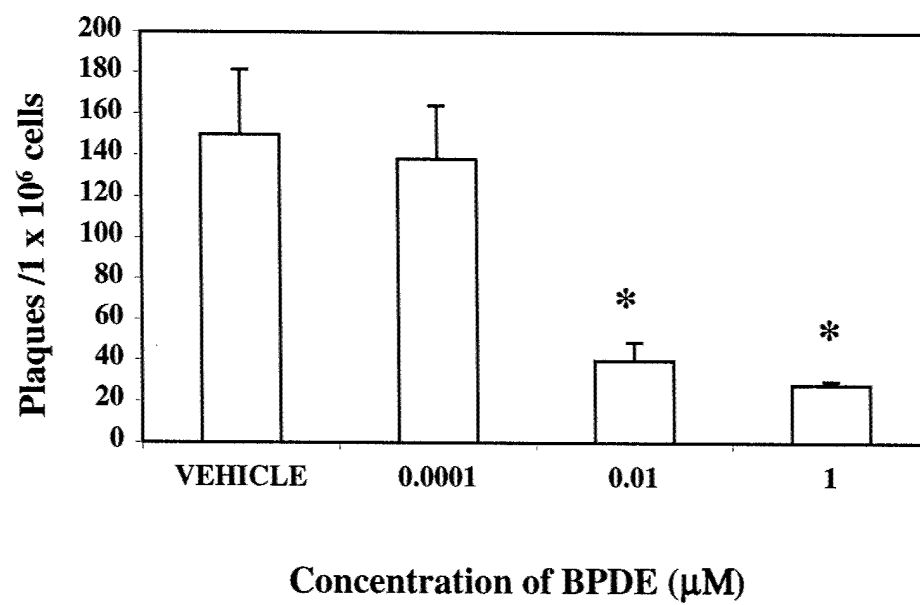


Figure 36. Analysis of CYP1A protein expression and activity in isolated kidney mononuclear cells 24 h following *in vitro* exposure to BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either vehicle or BPDE at 0.0001, 0.01, 1.0 μ M. Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells by CYP1A immunocytochemistry (ICC) 24 h post-culture initiation, and data was expressed as either (A) the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive; $n = 3$ experiments $\pm$ S.E.M.) or (B) the mean frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index; $n = 3$ experiments $\pm$ S.E.M.). The activity of CYP1A within adherent and non-adherent cell populations was determined by an *in vitro* EROD assay and data was expressed as (C) the mean pmoles of resorufin /min /mg protein ($n = 3$ experiments $\pm$ S.E.M.). *# Significantly different from the vehicle or 0.0001 μ M BPDE exposure group within the designated cell population ($p \leq 0.05$), respectively.

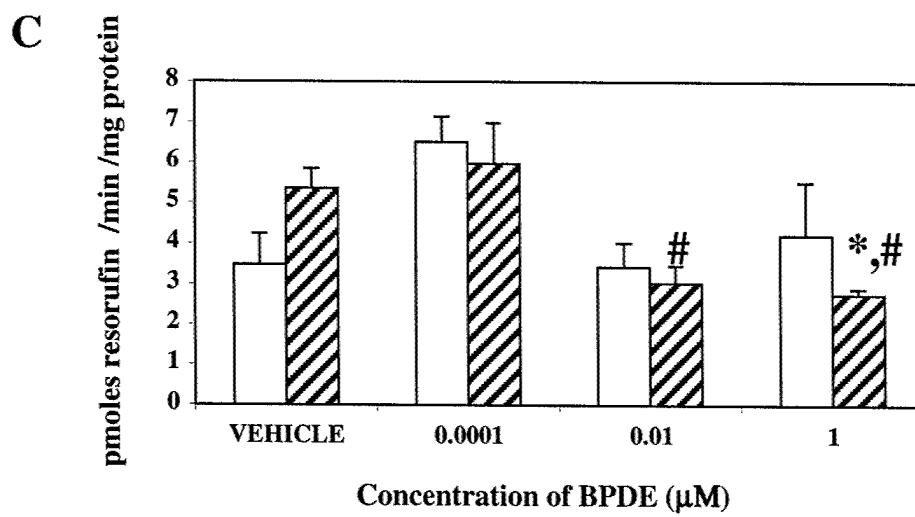
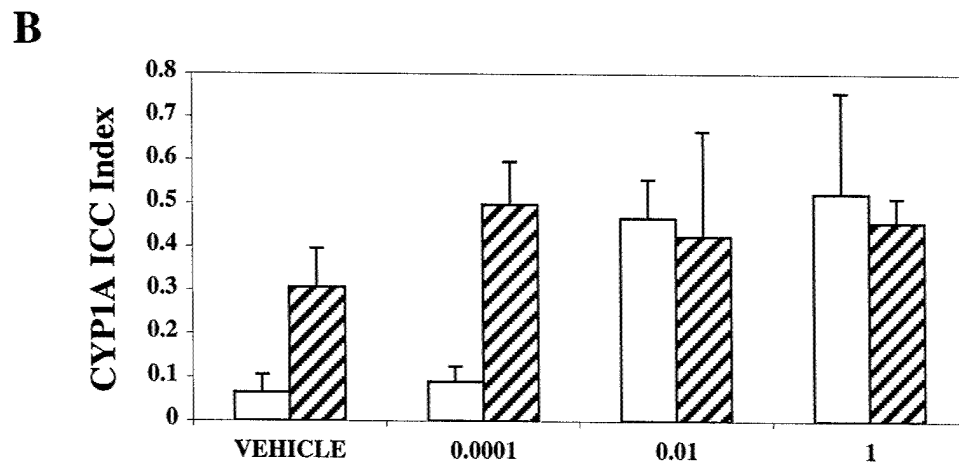
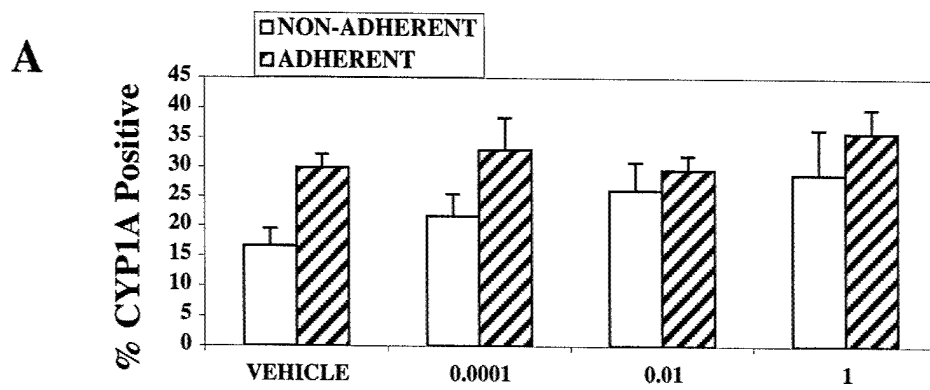
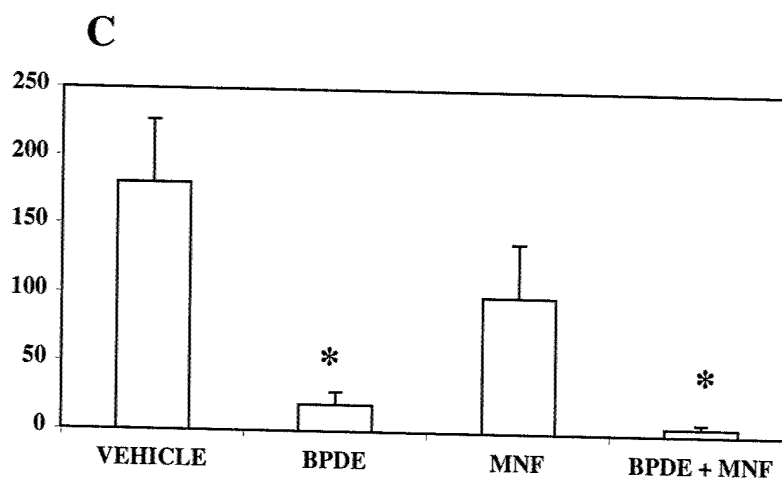
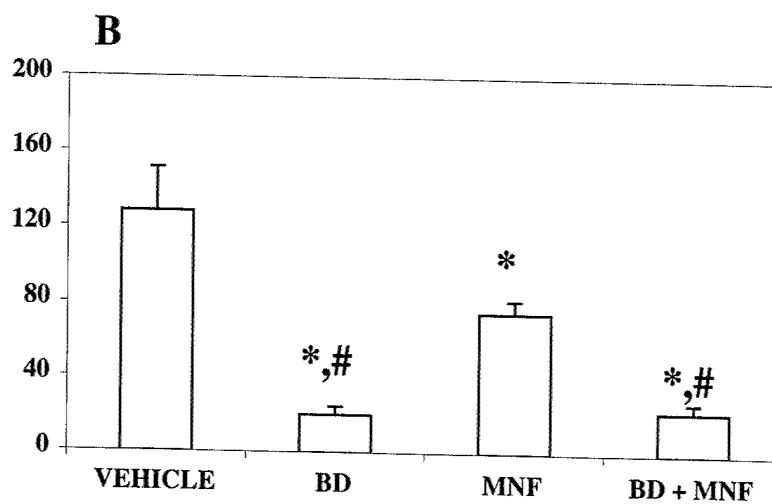
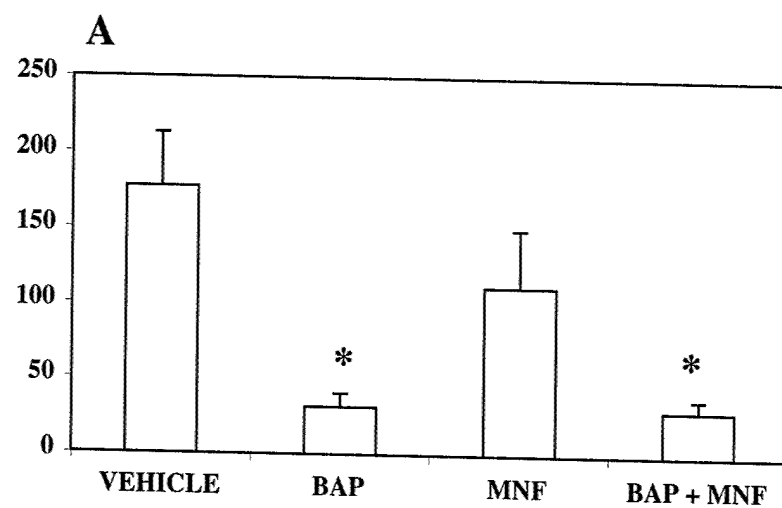


Figure 37. Medaka PFC numbers following *in vitro* exposure to 3'-methoxy-4'-nitroflavone (MNF) in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M of BaP or its metabolites (either BD or BPDE), 0.05 μ M MNF, BaP or its metabolites + MNF (1.0 μ M and 0.05 μ M, respectively), or vehicle, and subsequently immunized with SRBCs. Nine days post-culture initiation, PFCs were enumerated and data expressed as mean number of PFCs / 1×10^6 cells for (A) BaP, (B) BD, or (C) BPDE studies ($n = 3$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the MNF exposure group ($p \leq 0.05$).

Plaques / 1×10^6 cells



Chemical Treatment

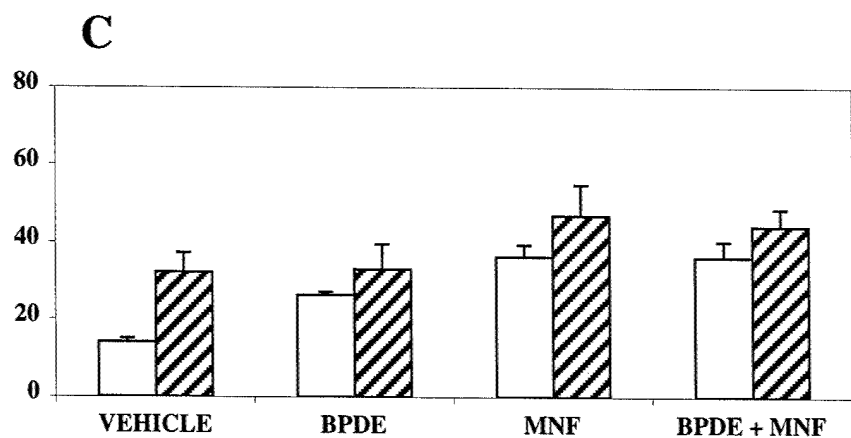
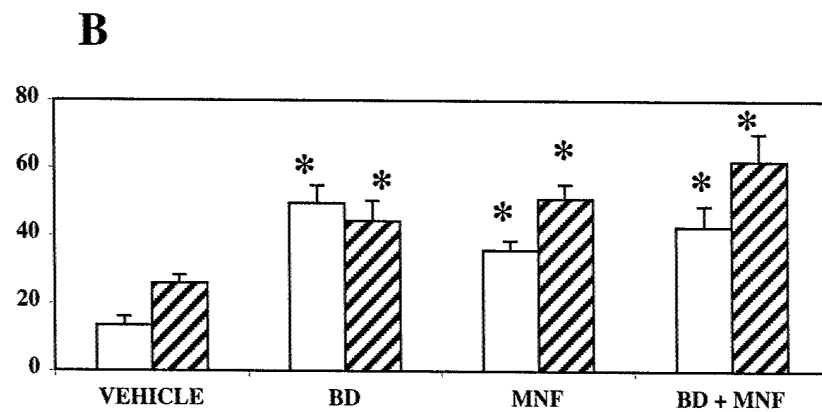
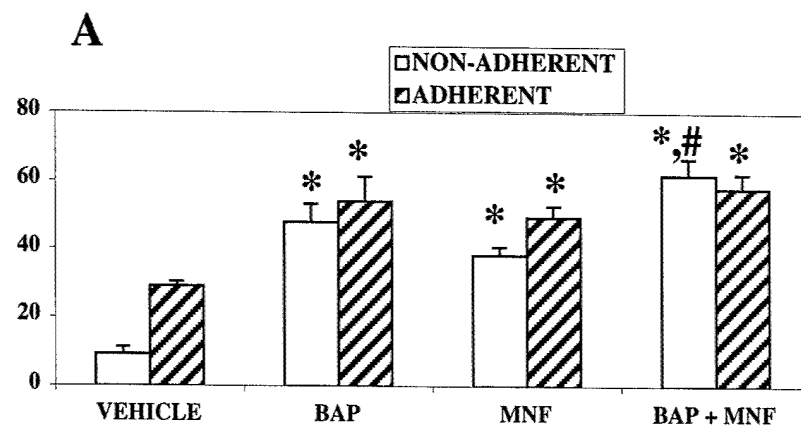
only MNF significantly reduced PFC numbers compared to vehicle control. Finally, treatment with 1.0 μ M BPDE alone or in combination with MNF significantly depressed PFC numbers compared to the vehicle control (Figure 37C).

The percentage of both adherent and non-adherent cells expressing CYP1A protein was significantly greater (compared to vehicle control) for cultures exposed to: 1.0 μ M BaP alone; 1.0 μ M BD alone; 0.05 μ M MNF alone; BaP + MNF; and, BD + MNF (Figure 38A & B). In addition, exposure to BaP + MNF significantly increased the percentage of CYP1A-positive non-adherent cells compared to cultures exposed to MNF alone. No significant differences in percentages of CYP1A-positive cells were observed between cells exposed to the vehicle control, 1.0 μ M BPDE, or a combination of both 1.0 μ M BPDE and 0.05 μ M MNF (Figure 38C).

Significantly higher CYP1A ICC Indices were observed in adherent and non-adherent cells exposed to 1.0 μ M BaP compared to vehicle control (Figure 39A). Exposure to MNF alone and BaP + MNF also resulted in significantly higher adherent cell CYP1A ICC Indices compared to the vehicle control. Although 1.0 μ M BD treatment did not alter CYP1A ICC Index, exposure to only MNF and BD + MNF significantly increased adherent cell CYP1A ICC Indices compared to vehicle control or the BD alone treatment groups (Figure 39B). In addition, treatment with BD + MNF resulted in a significantly higher CYP1A ICC Index in non-adherent cells compared to the vehicle control. For the BPDE exposure studies, exposure to both MNF alone and BPDE + MNF significantly increased adherent cell CYP1A ICC Indices compared to the vehicle control or BPDE treatment groups (Figure 39C).

Figure 38. Percentage of medaka mononuclear cells expressing CYP1A protein following *in vitro* exposure to MNF in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μM BaP or its metabolites (either BD or BPDE), 0.05 μM MNF, BaP or its metabolites + MNF (1.0 μM and 0.05 μM , respectively), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive) for (A) BaP, (B) BD, or (C) BPDE ($n = 3$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the MNF exposure group ($p \leq 0.05$).

% CYP1A Positive

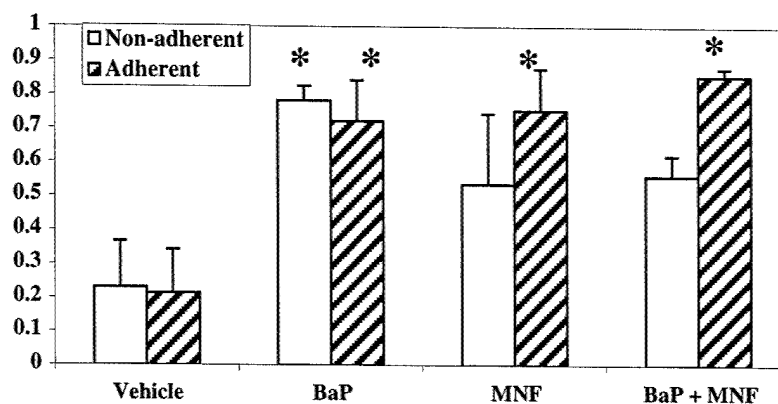


Chemical Treatment

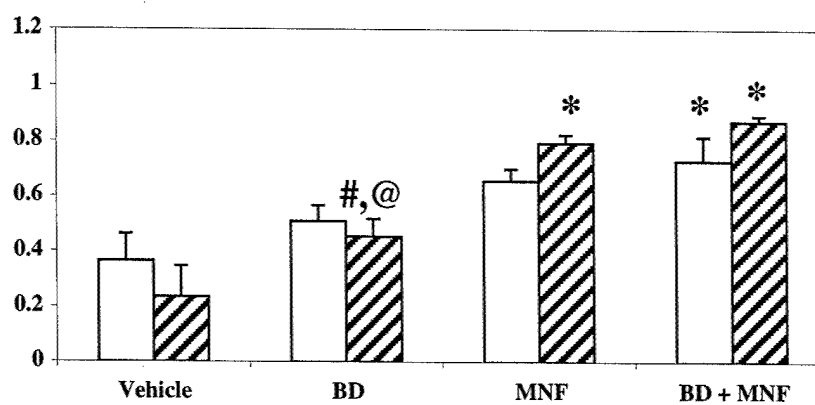
Figure 39. Medaka mononuclear cell CYP1A ICC Indices following *in vitro* exposure to MNF in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M of BaP or its metabolites (either BD or BPDE), 0.05 μ M MNF, BaP or its metabolites + MNF (1.0 μ M and 0.05 μ M, respectively), or vehicle and then analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index) for (A) BaP, (B) BD, or (C) BPDE ($n = 3$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). # Significantly different from the MNF exposure group ($p \leq 0.05$). @Significantly different from the BaP + MNF or BaP metabolite + MNF exposure group ($p \leq 0.05$).

CYP1A ICC Index

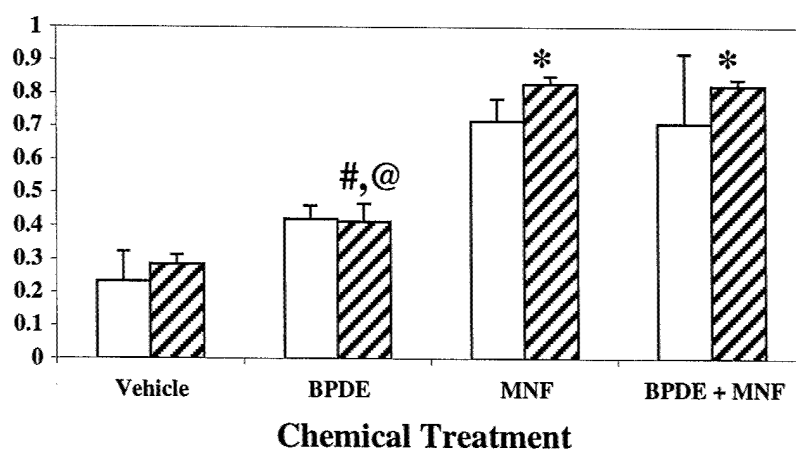
A



B



C



Significantly increased EROD activities in non-adherent cells (compared to vehicle control) were observed following exposure to BaP, BD, MNF, BaP + MNF, and BD + MNF (Figure 40A & B). CYP1A enzyme activity was also significantly higher in adherent cells exposed to BaP, BD, BaP + MNF, and BD + MNF (compared to vehicle control). For the BPDE exposure studies, exposure to MNF significantly increased EROD activity in non-adherent cells compared to cells treated with the vehicle control or BPDE alone (Figure 40C). Non-adherent cells exposed to BPDE + MNF also had significantly higher EROD activities than the vehicle control or BPDE only treatment groups.

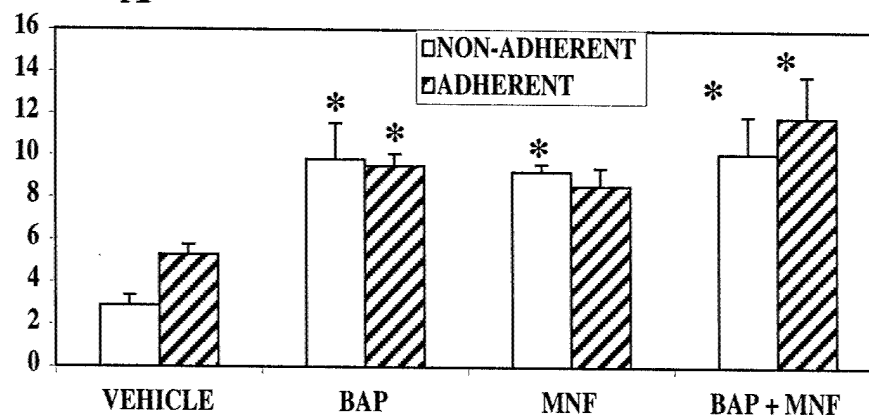
e. Effects of Concurrent In Vitro Exposure to BaP /BaP metabolites and α -Naphthoflavone (ANF) on Immune Function and CYP1A Expression /Activity

Exposure of cells to either BaP (Figure 41A) or BD (Figure 41B) significantly decreased PFC numbers compared to vehicle control, ANF alone, or individual BaP compound (i.e., BaP or BD) + ANF treatment groups. No differences in PFC numbers (compared to vehicle control) were observed following cell treatment with either ANF alone, BaP + ANF, or BD + ANF. Both BPDE alone and BPDE + ANF exposure groups demonstrated significantly fewer numbers of PFCs compared to that observed in the vehicle control and ANF treatment groups (Figure 41C).

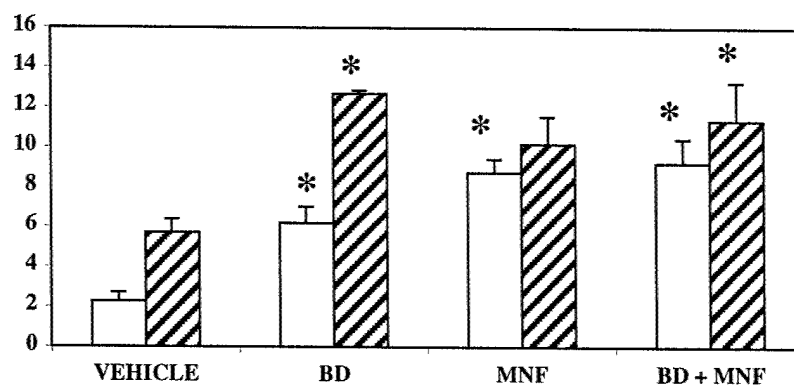
Figure 40. Medaka mononuclear cell CYP1A enzyme activity following *in vitro* exposure to MNF in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M of BaP or its metabolites (either BD or BPDE), 0.05 μ M MNF, BaP or its metabolites + MNF (1.0 μ M and 0.05 μ M, respectively), or vehicle and then analyzed 24 h later for CYP1A activity using an *in vitro* EROD assay. The activity of CYP1A within adherent and non-adherent cell populations was determined by an *in vitro* EROD assay and data was expressed as the mean pmoles of resorufin /min /mg protein for (A) BaP, (B), BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). # Significantly different from the MNF exposure group ($p \leq 0.05$). @Significantly different from the BaP + MNF or BaP metabolite + MNF exposure group ($p \leq 0.05$).

pmoles resorufin /min /mg protein

A



B



C

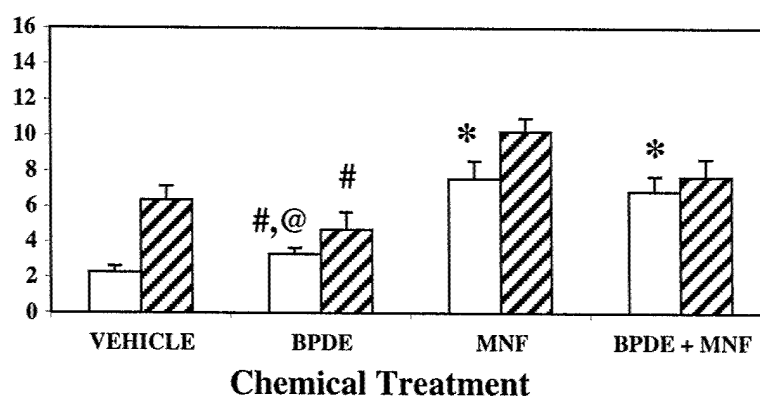
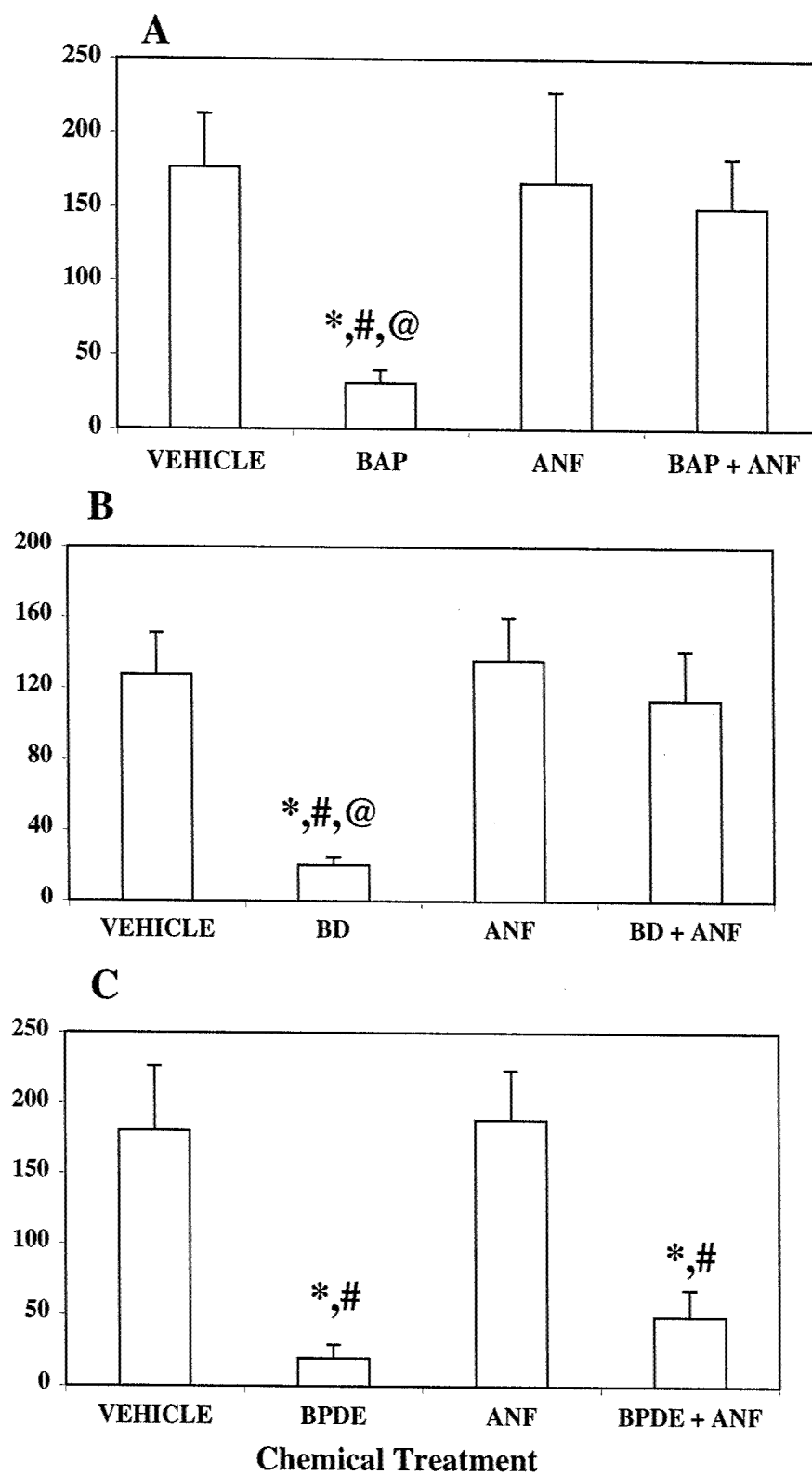


Figure 41. Medaka PFC numbers following *in vitro* exposure to α -naphthoflavone (ANF) in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 1.0 μ M ANF, BaP or its metabolites + ANF (1.0 μ M each), or vehicle and subsequently immunized with SRBCs. Nine days post-culture initiation, PFCs were enumerated and data expressed as the mean number of specific PFCs / 1×10^6 cells for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the ANF exposure group ($p \leq 0.05$). @Significantly different from the BaP + ANF or BaP metabolite + ANF exposure group ($p \leq 0.05$).

Plaques / 1×10^6 cells



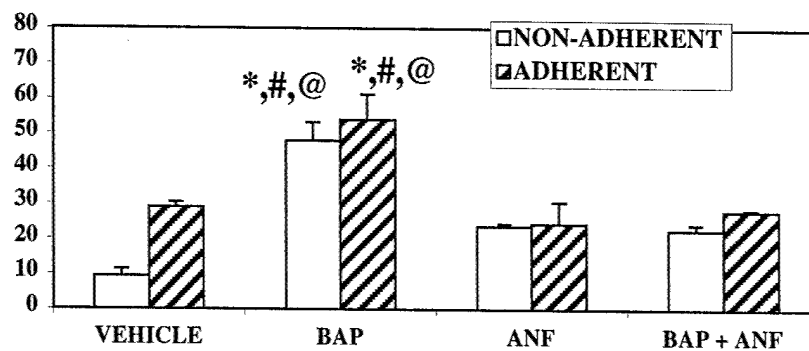
A significantly greater percentage of adherent and non-adherent cells expressed CYP1A protein following exposure to 1.0 μ M BaP compared to treatment with the vehicle control, ANF, or BaP + ANF (Figure 42A). Exposure of cells to 1.0 μ M BD significantly increased the percentage of CYP1A-positive adherent and non-adherent cells compared to vehicle control cells and cells treated with ANF alone (Figure 42B). In addition, the BD alone exposure group possessed a significantly greater percentage of non-adherent cells expressing CYP1A compared to the BD + ANF treatment group; no differences were observed between vehicle control and any BPDE treatment groups (Figure 42C). For the BaP, BD, and BPDE studies, only exposure to 1.0 μ M BaP alone significantly increased CYP1A ICC Index (for both adherent and non-adherent cells) compared to the vehicle control (Figure 43).

Both adherent and non-adherent cells exposed to BaP (Figure 44A) or BD (Figure 44B) demonstrated significantly higher EROD activities compared to the vehicle control, ANF, or BaP/BD + ANF treatment groups. For the BPDE studies, no significant differences in EROD activity were observed between any of the treatment groups and the vehicle control (Figure 44C).

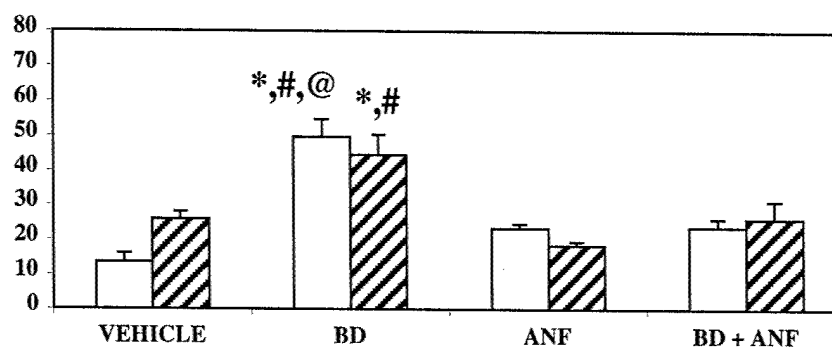
Figure 42. Percentage of medaka mononuclear cells expressing CYP1A protein following *in vitro* exposure to ANF in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M of BaP (or its metabolites BD or BPDE), 1.0 μ M ANF, BaP or its metabolites + ANF (1.0 μ M each), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive) for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the ANF exposure group ($p \leq 0.05$). @Significantly different from the BaP + ANF or BaP metabolite + ANF exposure group ($p \leq 0.05$).

% CYP1A Positive

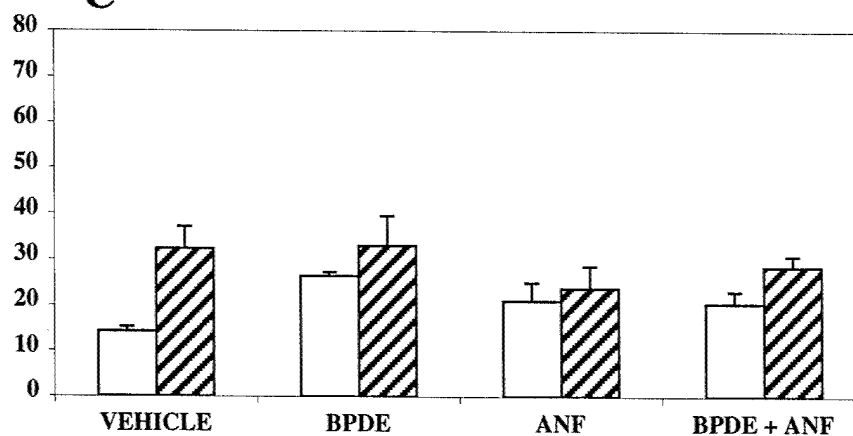
A



B



C



Chemical Treatment

Figure 43. Medaka mononuclear cell CYP1A ICC Indices following *in vitro* exposure to ANF in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M of BaP or its metabolites (either BD or BPDE), 1.0 μ M ANF, BaP or its metabolites + ANF (1.0 μ M each), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index) for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$).

CYP1A ICC Index

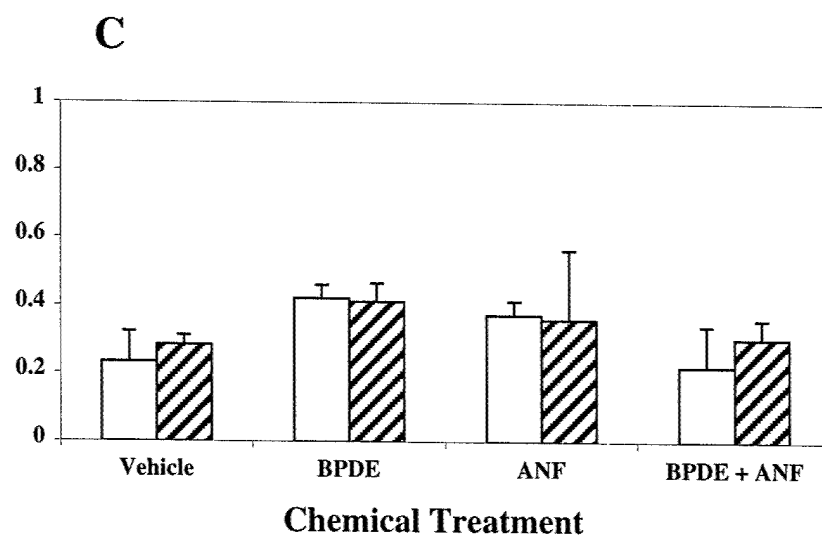
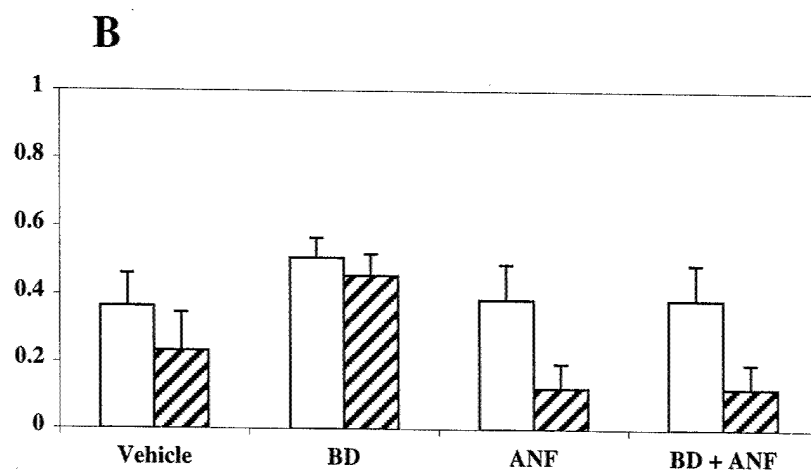
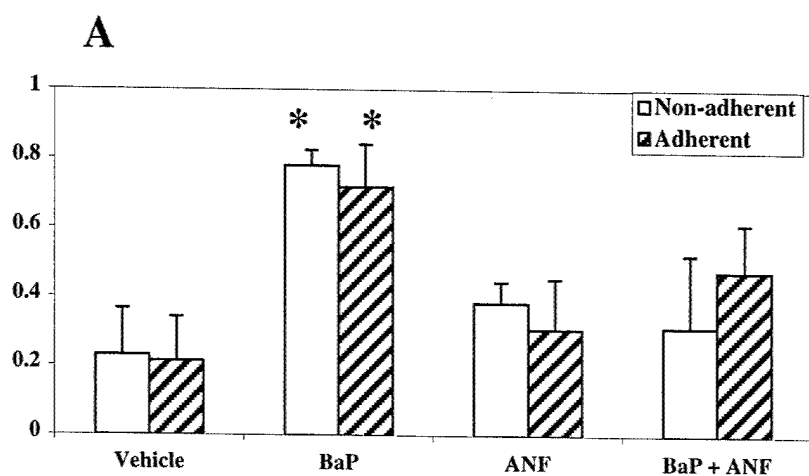
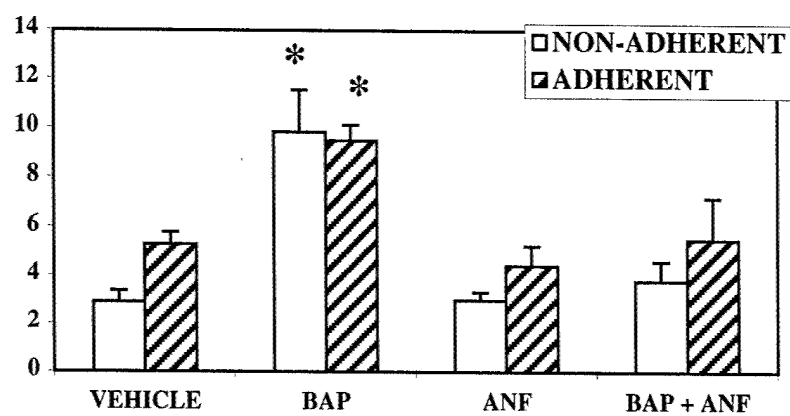


Figure 44. Medaka mononuclear cell CYP1A enzyme activity following *in vitro* exposure to ANF in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M of BaP (or its metabolites BD or BPDE), 1.0 μ M ANF, BaP or its metabolites + ANF (1.0 μ M each), or vehicle and analyzed 24 h later for CYP1A activity using an *in vitro* EROD assay. Activity of CYP1A within adherent and non-adherent cell populations was determined using an *in vitro* EROD assay; data was the expressed as the mean pmoles of resorufin /min /mg protein for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.).

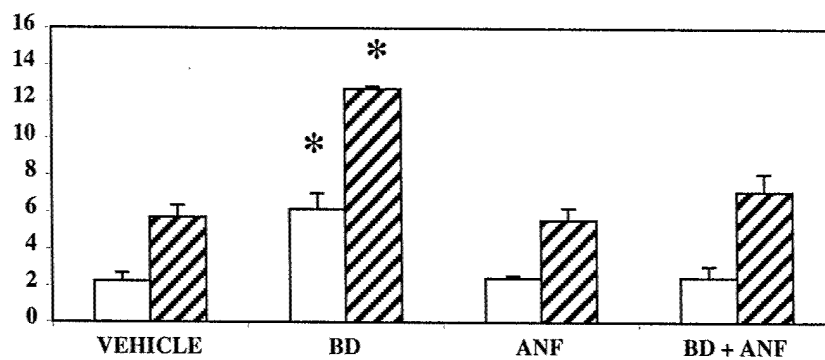
*Significantly different from vehicle control, ANF, and BaP +ANF or BaP metabolite + ANF exposure groups ($p \leq 0.05$).

pmoles resorufin /min /mg protein

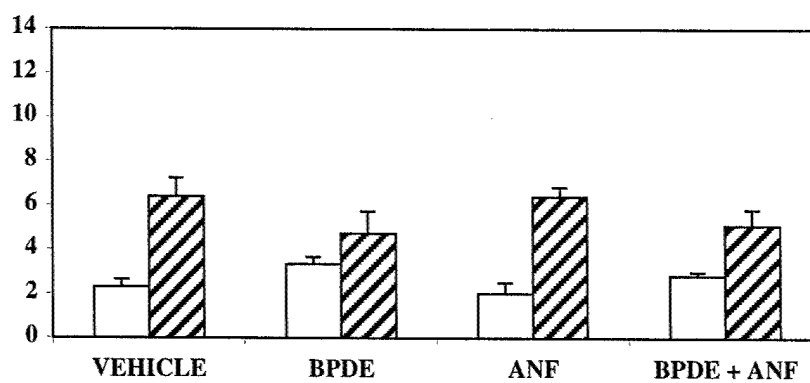
A



B



C



Chemical Treatment

f. Effects of Concurrent In Vitro Exposure to BaP /BaP

Metabolites and Dehydroepiandrosterone (DHEA) on Immune Function and CYP1A Expression /Activity

Treatment with both 1.0 μ M BaP and BaP + DHEA significantly reduced PFC numbers compared to vehicle control (Figure 45A). In addition, both the 1.0 μ M BD and BD + DHEA treatment groups demonstrated significantly fewer PFC numbers compared to the vehicle control (Figure 45B). Cells exposed to 1.0 μ M BD alone also demonstrated significantly lower PFC numbers than the DHEA treatment group. Exposure to either BPDE or BPDE + DHEA significantly reduced PFC numbers compared to the vehicle control (Figure 45C).

A significantly greater percentage of adherent and non-adherent cells expressing CYP1A protein was observed following treatment with either BaP or BD compared to cells treated with the vehicle control, DHEA, or BaP/BD + DHEA (Figure 46A & B). For the BPDE exposure studies, no difference were observed between any treatment group and the vehicle control (Figure 46C). Significantly higher CYP1A ICC Indices were observed for adherent and non-adherent cells exposed to 1.0 μ M BaP compared to the vehicle control (Figure 47A). The mean non-adherent cell CYP1A ICC Index for the BaP exposure group was also significantly higher than that for the DHEA and the BaP + DHEA treatment groups. For the BD studies, a significantly lower CYP1A ICC Index was exhibited by non-adherent cells exposed to DHEA alone compared to vehicle control, BaP alone, or BaP + DHEA

Figure 45. Medaka PFC numbers following *in vitro* exposure to dehydroepiandrosterone (DHEA) in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 1.0 μ M DHEA, BaP or its metabolites + DHEA (1.0 μ M each), or vehicle and subsequently immunized with sheep red blood cells. Nine days post-culture initiation, PFCs were enumerated and data was expressed as the mean number of specific PFCs / 1×10^6 cells for (A) BaP, (B) BD, or (C) BPDE ($n = 3$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the DHEA exposure group ($p \leq 0.05$).

Plaques / 1×10^6 cells

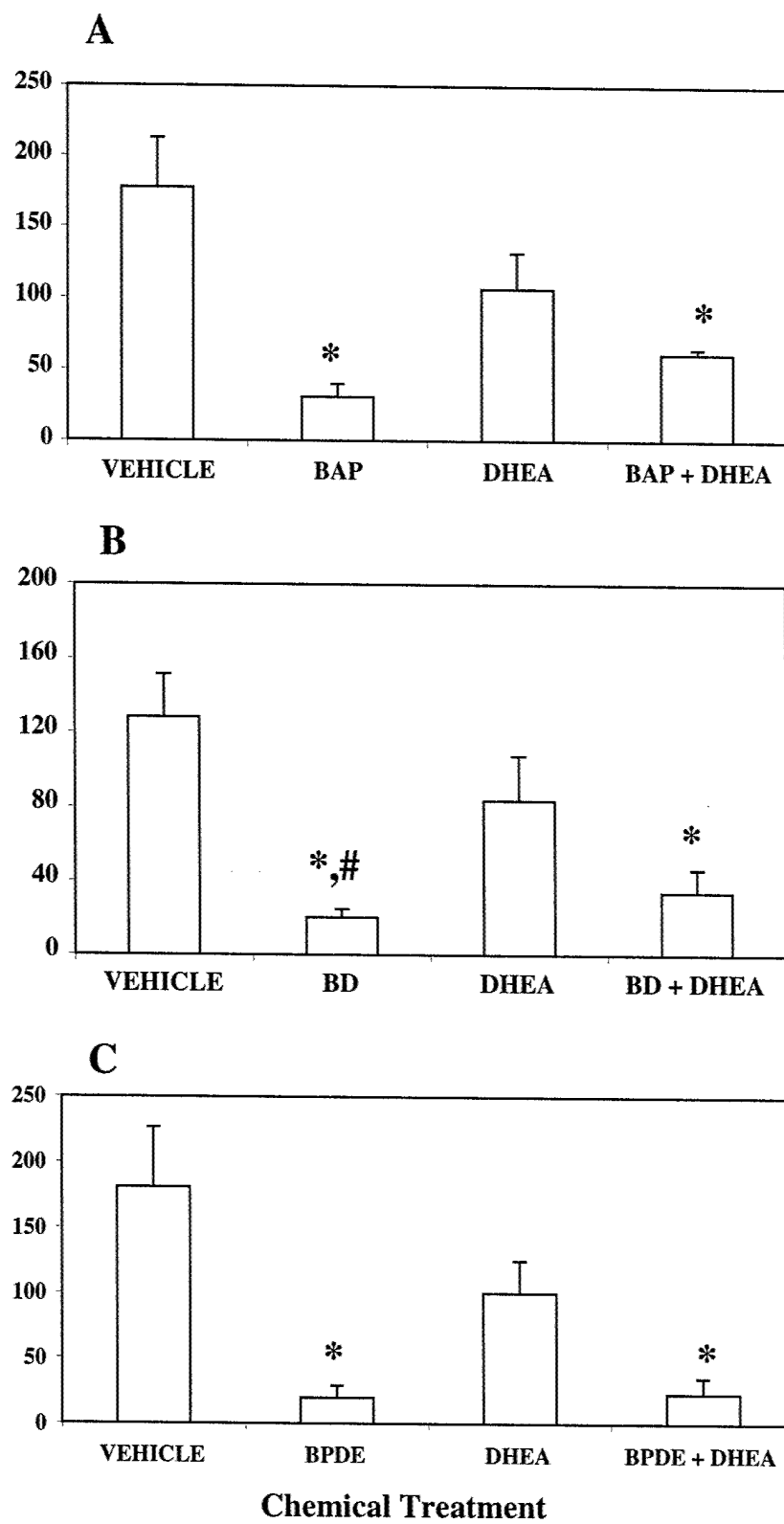
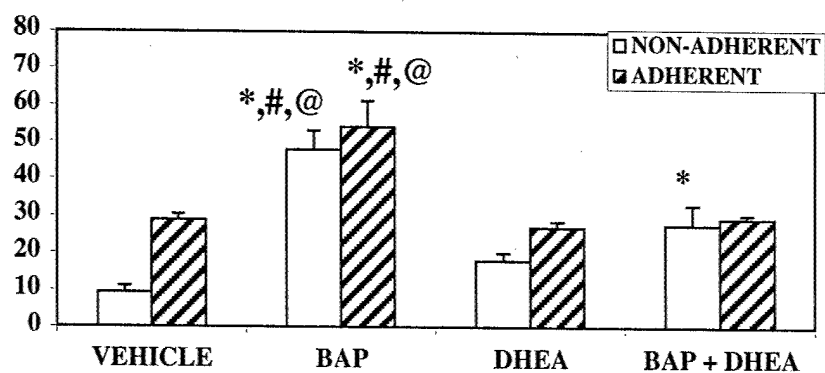


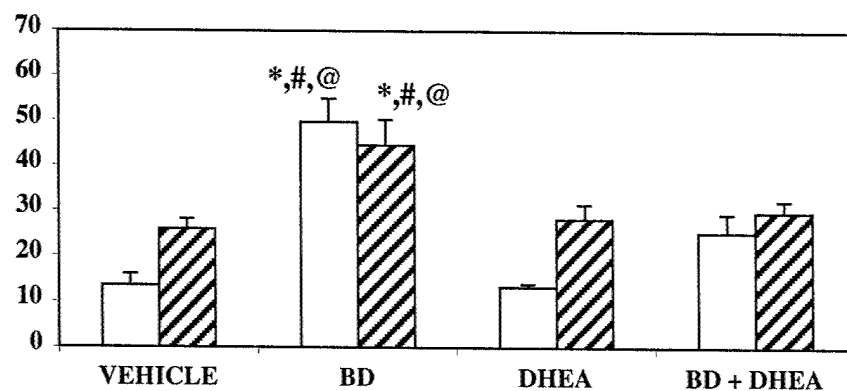
Figure 46. Percentage of medaka mononuclear cells expressing CYP1A protein following *in vitro* exposure to DHEA in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 1.0 μ M DHEA, BaP or its metabolites + DHEA (1.0 μ M each), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive) for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the DHEA exposure group ($p \leq 0.05$). @Significantly different from the BaP + DHEA or BaP metabolite + DHEA exposure group ($p \leq 0.05$).

% CYP1A Positive

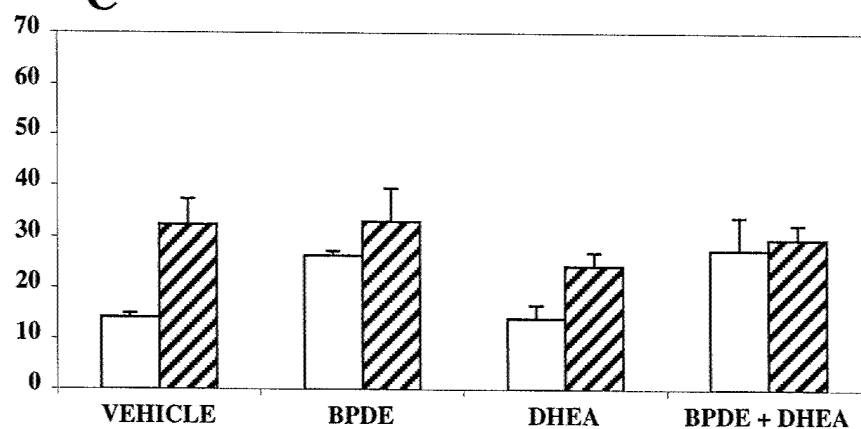
A



B



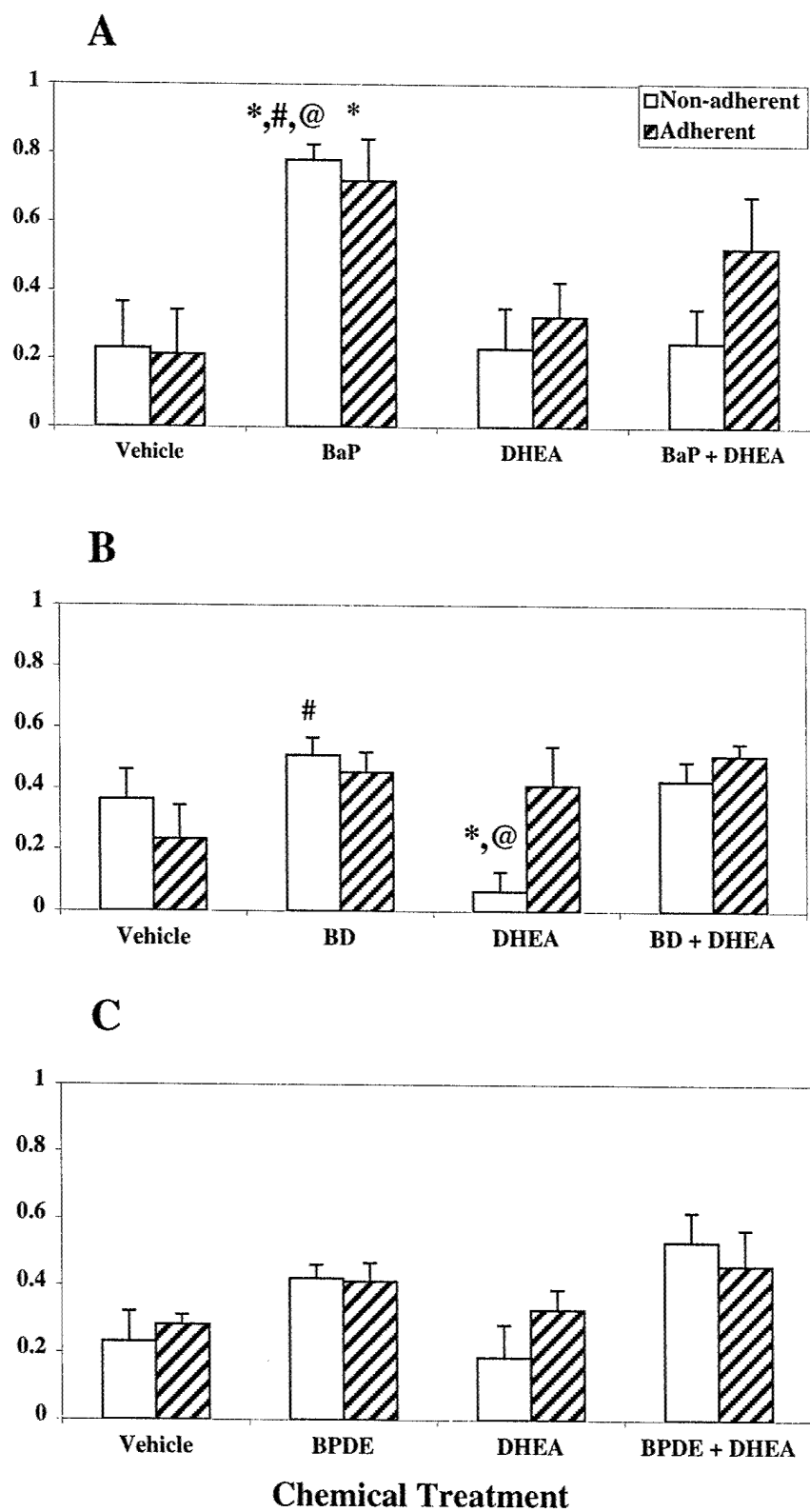
C



Chemical Treatment

Figure 47. Medaka mononuclear cell CYP1A ICC Indices following *in vitro* exposure to DHEA in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 1.0 μ M DHEA, BaP or its metabolites + DHEA (1.0 μ M each), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index) for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the DHEA exposure group ($p \leq 0.05$). ®Significantly different from the BaP + DHEA or BaP metabolite + DHEA exposure group ($p \leq 0.05$).

CYP1A ICC Index



(Figure 47B). No significant differences were observed between vehicle control and any BPDE treatment groups (Figure 47C).

Significantly higher EROD activities were seen for both adherent and non-adherent cells following 1.0 μ M BaP treatment compared to vehicle control and the DHEA treatment group (Figure 48A). Adherent cells from the BaP treatment group also demonstrated significantly higher EROD activity compared to cells exposed to BaP + DHEA. In addition, both adherent and non-adherent cells exposed to 1.0 μ M BD demonstrated significantly elevated EROD activities compared to cells treated with either vehicle control, DHEA, or BD + DHEA (Figure 48B); no significant differences in EROD activity was observed between the vehicle control and any groups exposed to BPDE (Figure 48C).

g. Effect of Concurrent In Vitro Exposure to BaP /BaP

Metabolites and Ellipticine (ELP) on Immune Function and CYP1A Expression /Activity

Exposure of cells to 1.0 μ M BaP significantly reduced PFC numbers compared to the vehicle control (Figure 49A). In addition, BaP-exposed cultures also demonstrated PFC numbers only slightly lower than cultures treated with either ELP or BaP + ELP. Significantly fewer PFCs were observed in cultures following exposure to 1.0 μ M BD compared to the vehicle control, ELP, or BD + ELP treatment groups (Figure 49B). Exposure to either BPDE alone or in combination with ELP

Figure 48. Medaka mononuclear cell CYP1A enzyme activity following *in vitro* exposure to DHEA in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 1.0 μ M DHEA, BaP or its metabolites + DHEA (1.0 μ M each), or vehicle and analyzed 24 h later for CYP1A activity using an *in vitro* EROD assay. The activity of CYP1A within adherent and non-adherent cell populations was determined using an *in vitro* EROD assay and data was expressed as the mean pmoles of resorufin /min /mg protein for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.).

*Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the DHEA exposure group ($p \leq 0.05$). @Significantly different from the BaP + DHEA or BaP metabolite + DHEA exposure group ($p \leq 0.05$).

pmoles resorufin /min /mg protein

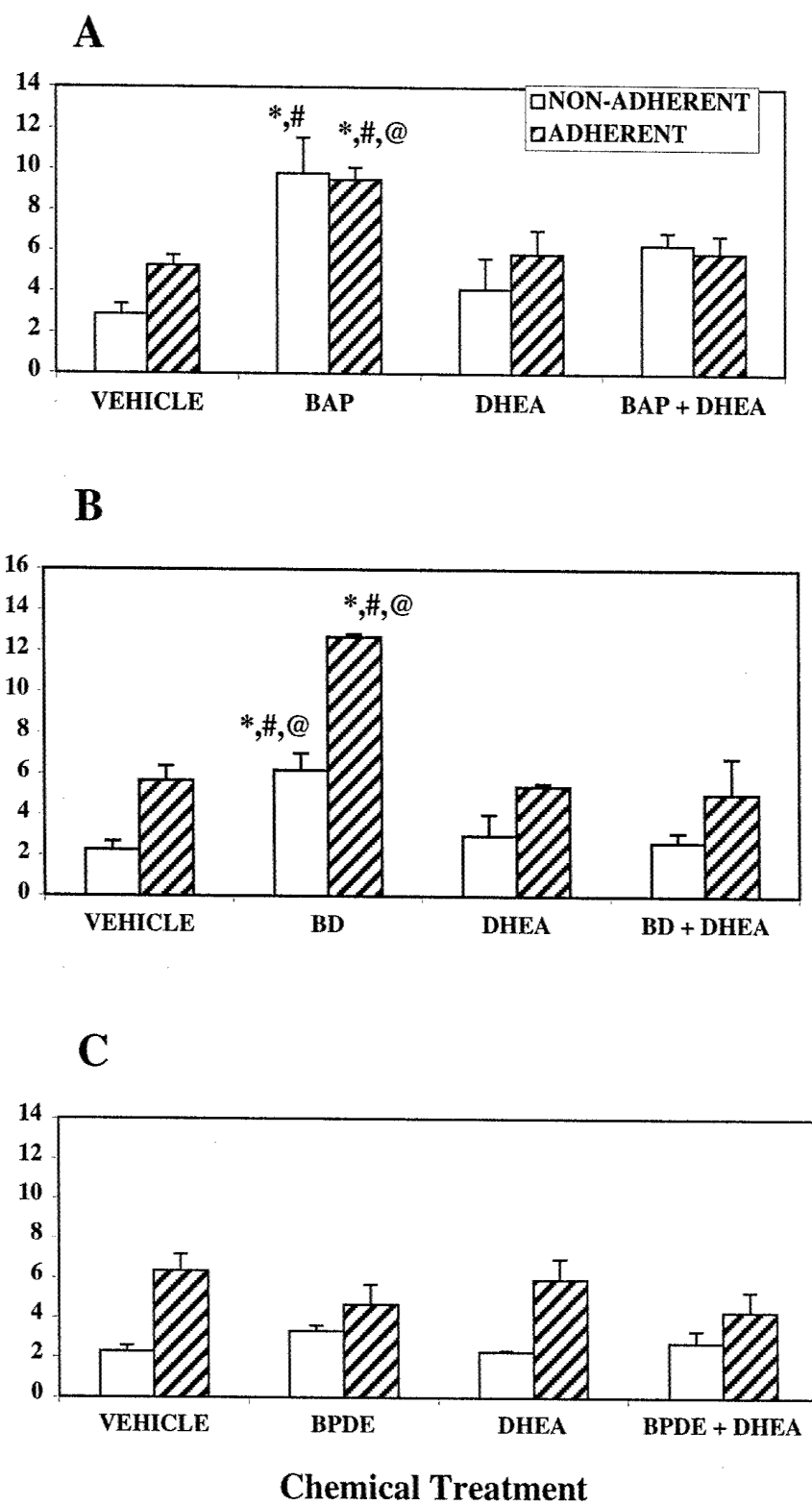
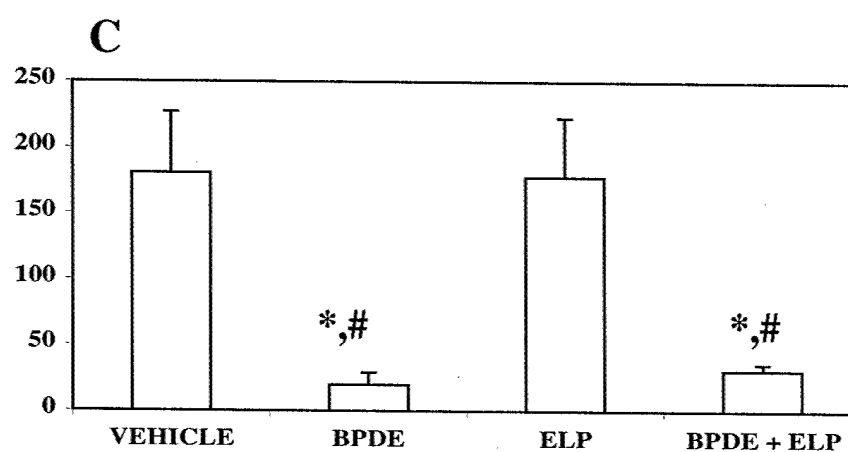
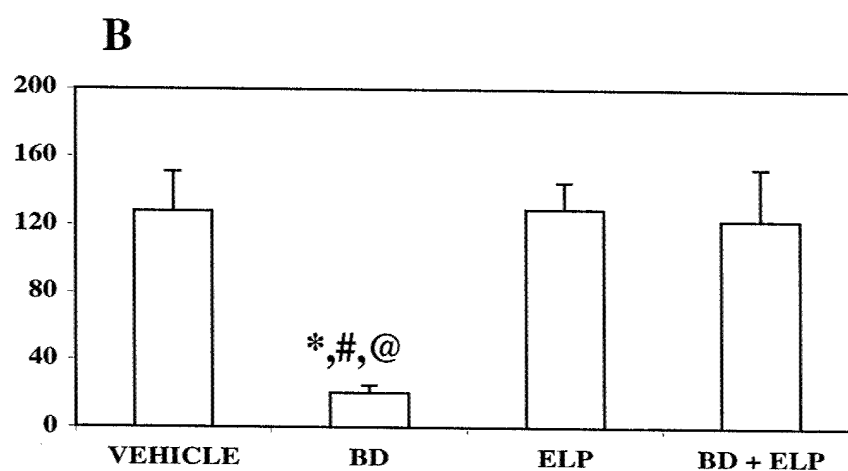
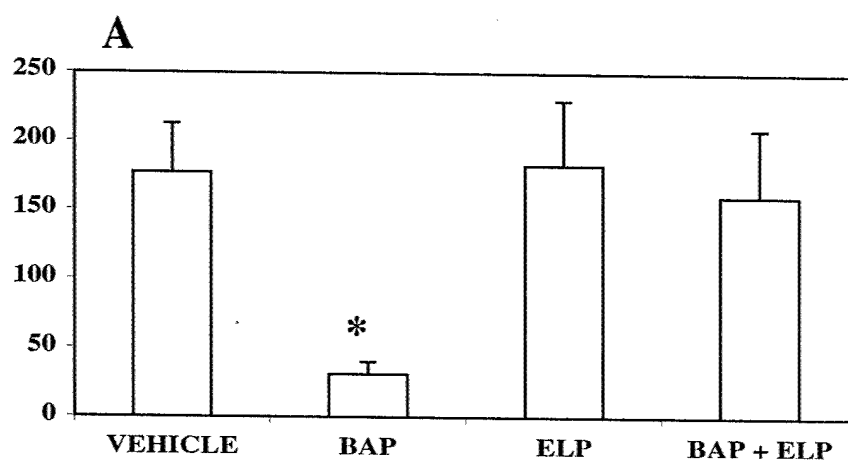


Figure 49. Medaka PFC numbers following *in vitro* exposure to ellipticine (ELP) in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μM BaP (or its metabolites BD or BPDE), 0.0001 μM ELP, BaP or its metabolites + ELP (1.0 μM and 0.0001 μM , respectively), or vehicle and subsequently immunized with SRBCs. Nine days post-culture initiation, PFCs were enumerated and data was expressed as the mean number of PFCs / 1×10^6 cells for (A) BaP, (B) BD, or (C) BPDE ($n = 3$ experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the ELP exposure group ($p \leq 0.05$). @Significantly different from the BaP + ELP or BaP metabolite + ELP exposure group ($p \leq 0.05$).

Plaques / 1×10^6 cells



Chemical Treatment

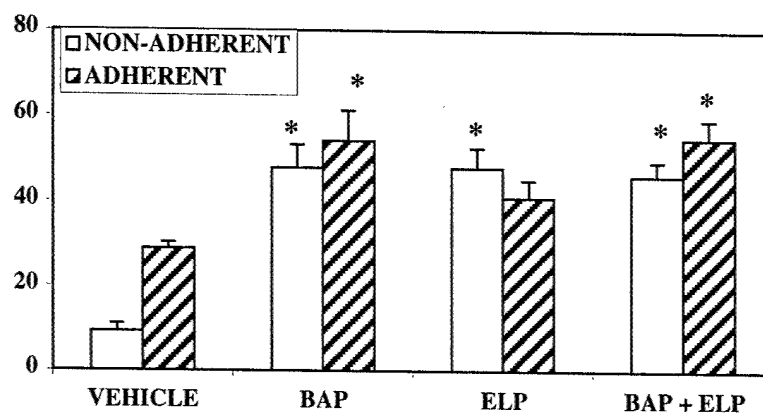
significantly reduced PFC numbers compared to the vehicle control and ELP treatment groups (Figure 49C).

Exposure of cells to BaP, ELP, or both chemicals in combination resulted in a significantly higher percentage of non-adherent cells expressing CYP1A protein compared to the vehicle control (Figure 50A). The percentage of CYP1A-positive adherent cells was also significantly higher for cells treated with BaP or BaP + ELP compared to vehicle control. A greater number of CYP1A-positive non-adherent cells was observed following exposure to BD, ELP, and BD + ELP compared to vehicle control (Figure 50B); no differences in the percentage of adherent CYP1A-positive cells were observed between vehicle control and any BD treatment groups. Both ELP alone and in combination with BPDE treatment groups demonstrated significantly higher percentages of non-adherent CYP1A-positive cells compared to the vehicle control (Figure 50C). Exposure of cells to BaP, ELP, and BaP in combination with ELP significantly increased adherent cell CYP1A ICC Indices compared to vehicle control (Figure 51A); only cells treated with BaP demonstrated a significantly higher non-adherent cell CYP1A ICC Index (compared to vehicle control). Both adherent and non-adherent cells exposed to either ELP or ELP in combination with BD exhibited significantly greater CYP1A ICC Indices compared to the vehicle control (Figure 51B). The CYP1A ICC Index of adherent cells exposed to BD + ELP was also significantly greater than that of the BD exposure group. Adherent and non-adherent cells exposed to either ELP or ELP in combination with BPDE had significantly higher CYP1A ICC Indices compared to the vehicle control (Figure 51C). CYP1A

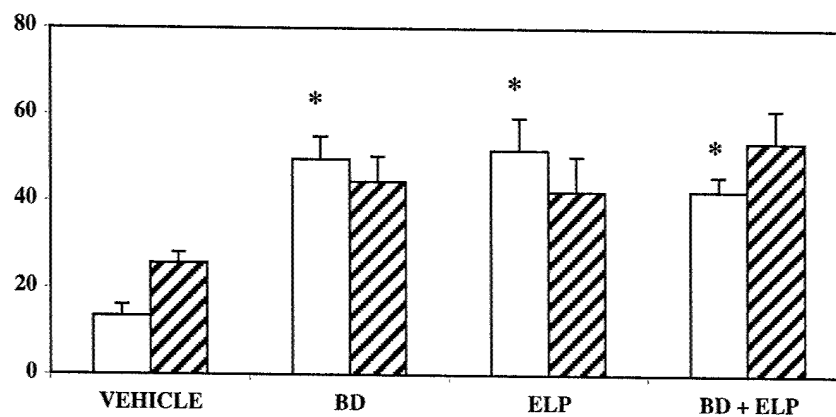
Figure 50. Percentage of medaka mononuclear cells expressing CYP1A protein following *in vitro* exposure to ELP in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 0.0001 μ M ELP, BaP or its metabolites + ELP (1.0 μ M and 0.0001 μ M, respectively), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed for adherent and non-adherent mononuclear cells and calculated as the mean percentage of cells staining positive for CYP1A (i.e., % CYP1A Positive) for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$).

% CYP1A Positive

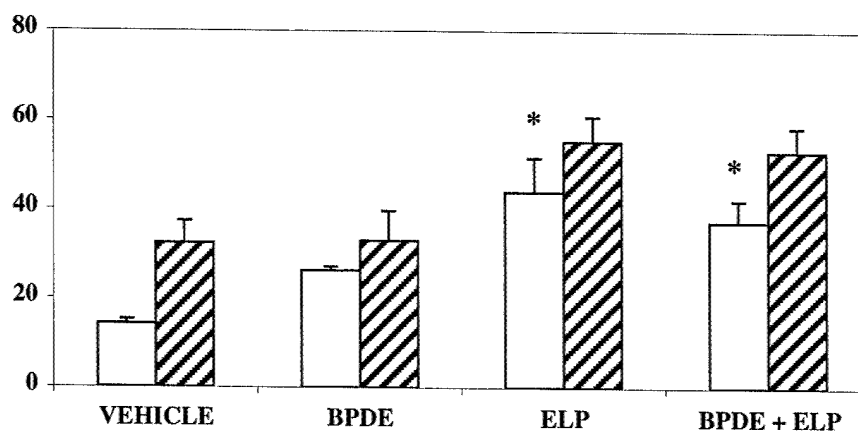
A



B



C



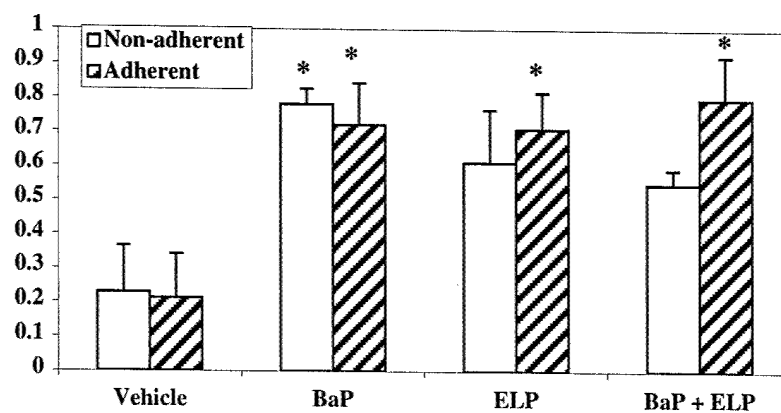
Chemical Treatment

Figure 51. Medaka mononuclear cell CYP1A ICC Indices following *in vitro* exposure to ELP in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 0.0001 μ M ELP, BaP or its metabolites + ELP (1.0 μ M and 0.0001 μ M, respectively), or vehicle and analyzed 24 h later for CYP1A expression by immunocytochemistry (ICC). Expression of CYP1A protein was assessed within adherent and non-adherent mononuclear cells and expressed as the mean relative frequency of cells expressing moderate to intense staining $\div$ by the number of positively-staining cells (i.e., CYP1A ICC Index) for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.).

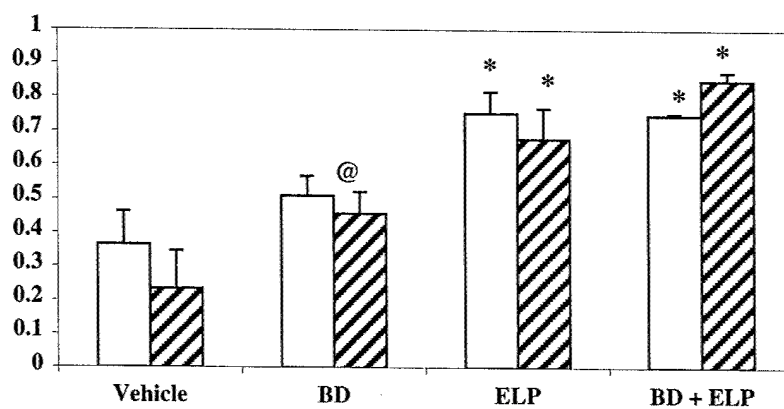
*Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the ELP exposure group ($p \leq 0.05$). @Significantly different from the BaP + ELP or BaP metabolite + ELP exposure group ($p \leq 0.05$).

CYP1A ICC Index

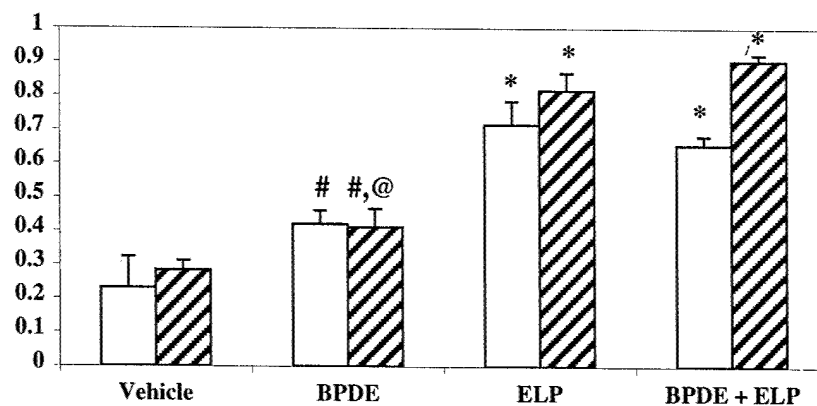
A



B



C



Chemical Treatment

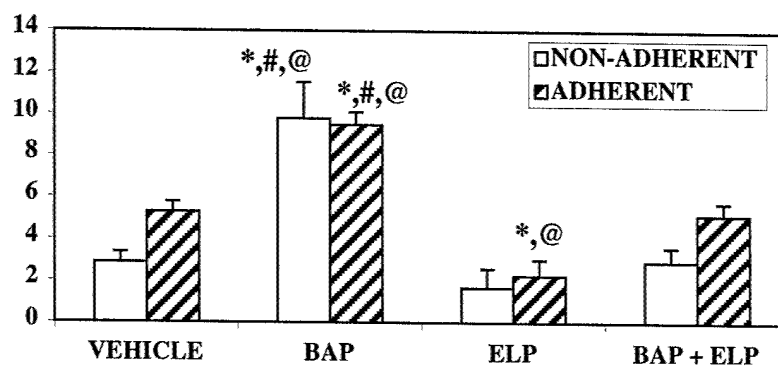
ICC Indices of both adherent and non-adherent cells exposed to ELP were also significantly greater than that of the BPDE treatment group; adherent cells exposed to BPDE + ELP also had significantly greater indices than the BPDE treatment group.

Exposure of cells to both BaP and BD significantly increased EROD activity for both adherent and non-adherent cells compared to the vehicle control, ELP, and BaP/BD + ELP treatment groups (Figure 52A and B). Adherent cells exposed to ELP demonstrated significantly lower EROD activities than the vehicle control, BaP + ELP, or BD + ELP treatment groups. Exposure of cells to BPDE in combination with ELP significantly reduced EROD activity of both the adherent and non-adherent cell populations compared to the vehicle control (Figure 52C).

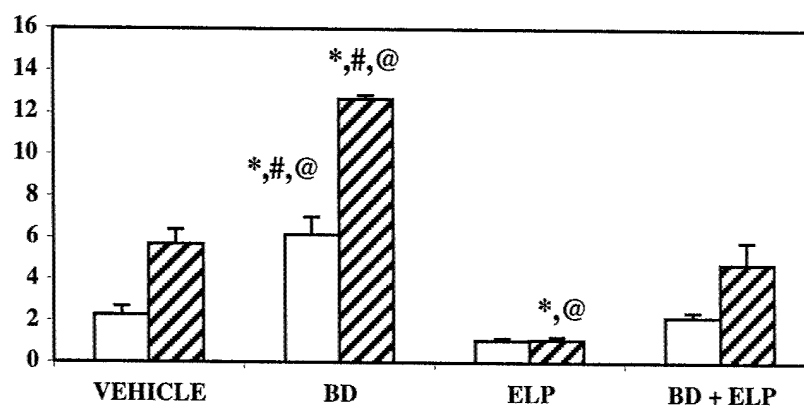
Figure 52. Medaka mononuclear cell CYP1A enzyme activity following *in vitro* exposure to ELP in combination with BaP, BD, or BPDE. Isolated kidney mononuclear cells were exposed *in vitro* to either 1.0 μ M BaP (or its metabolites BD or BPDE), 0.0001 μ M ELP, BaP or its metabolites + ELP (1.0 μ M and 0.0001 μ M, respectively), or vehicle and then analyzed 24 h later for CYP1A activity by an *in vitro* EROD assay. CYP1A activity within adherent and non-adherent cell populations was determined using an *in vitro* EROD assay; data were expressed as the mean pmoles of resorufin /min /mg protein for (A) BaP, (B) BD, or (C) BPDE (n = 3 experiments $\pm$ S.E.M.). *Significantly different from vehicle control ($p \leq 0.05$). #Significantly different from the ELP exposure group ($p \leq 0.05$). @Significantly different from the BaP + ELP or BaP metabolite + ELP exposure group ($p \leq 0.05$).

pmoles resorufin /min /mg protein

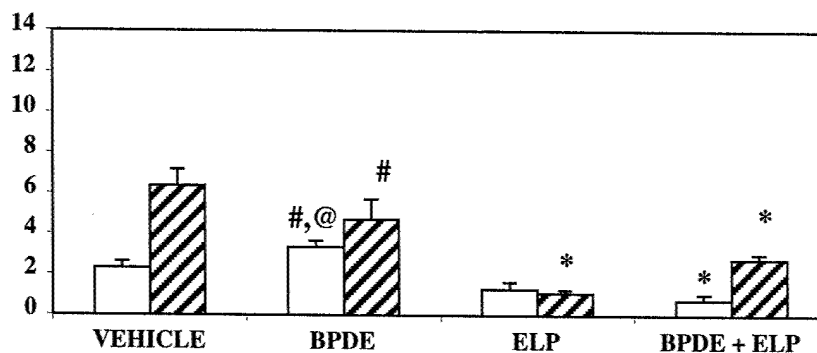
A



B



C



Chemical Treatment

VI. Discussion

A. Acute Exposure of Medaka to BaP Suppresses Immune Function & Host Resistance Against Bacterial Challenge

Although much is known regarding the carcinogenic properties of BaP in both mammals and fish, studies investigating the toxic effects of BaP upon the immune system have primarily been limited to rodent species (White et al., 1994). In rodents, the hallmark of BaP-induced immunotoxicity is its capacity to decrease numbers of T-dependent antigen-specific antibody forming cells. Benzo[a]pyrene-induced immunotoxic effects in rodents other than suppressed PFC numbers include: lymphoid organ atrophy (Holladay and Smith, 1995); suppressed mixed lymphocyte reactions and lymphocyte proliferation in response to mitogens (Dean et al., 1983; Urso et al., 1986; Urso, 1995; Mudzinski, 1993; Davila et al., 1996); altered T-cell differentiation (Rodriguez et al., 1999); decreased macrophage antigen-presenting capacity and cell-mediated immunity (Myers et al., 1987; Wojdani and Alfred, 1984); and, induction of contact hypersensitivity (Klemme et al., 1987; Anderson et al., 1995). In addition, BaP exposure has been found to alter cytokine profiles and lymphocyte calcium homeostasis (Lyte et al., 1987; Myers et al., 1988; Davila et al., 1995; Fujimaki et al., 1997; Mounho and Burchiel, 1998; Vandebriel et al., 1998).

Despite the wealth of information regarding the immunotoxicity of BaP in rodents, far less is known about its effects upon the immune system of aquatic species. Although altered immune function has been observed in feral fish sampled from aquatic environments heavily-contaminated with BaP or related PAHs, few laboratory

studies have been performed that attempt to identify those immune parameters most sensitive to BaP toxicity or the possible mechanisms by which these effects may have occurred (Weeks and Warinner, 1986; Weeks et al., 1987; Faisal et al., 1991; Pulsford et al., 1995; Arkoosh et al., 1991; 1998).

In this study, exposure of medaka to levels of BaP as high as 600 µg/g BW had no effect upon host survival or overall "good" health (as measured by changes in condition factors) in fish examined for up to 7d post-exposure. This observation coincides with data obtained in mammalian studies that demonstrated BaP not to be acutely toxic (EPA, 1990). Moreover, in the studies described herein, exposure to BaP had no effect upon thymus, kidney, and spleen cellularity or cell viability. While these latter results appear to contradict findings from previously-performed studies in rodents (Holladay and Smith, 1995) and in another teleost species (Holladay et al., 1998) which reported BaP-induced immune organ hypocellularity, these latter two studies employed multiple injections. For example, reductions of thymus, spleen, and bone marrow cellularity were observed in B₆C₃F₁ mice following five consecutive daily injections of 50 µg BaP/g BW (Holladay and Smith, 1995), and reduced pronephros cellularity was seen in tilapia (*Oreochromis niloticus*) after five consecutive daily injections of 5 µg BaP/g BW (Holladay et al., 1998). Since the study described herein examined immunotoxicity after only a single BaP injection, it is difficult to compare the current findings with those previously-reported in other species. Because BaP is rapidly metabolized and eliminated, multiple injection protocols may have resulted in a significantly greater contact time between BaP

(and/or its metabolites) and BaP-sensitive cell populations and, thus, accounted for the observed decrease in immune organ cellularity.

Exposure to 200 µg BaP/g BW significantly suppressed PMA-stimulated $\bullet\text{O}_2^-$ production by medaka kidney phagocytes. While the effect of IP administration of BaP upon phagocyte respiratory burst activity has not yet been reported in mammalian systems, Holladay et al. (1998) reported that five daily IP injections of BaP (final dose of 250 µg/g BW) suppressed PMA-stimulated H_2O_2 production in tilapia. Thus, innate immunity appears somewhat sensitive to the immunotoxic effects of BaP, though not to the same extent as humoral-related parameters.

At the relatively low concentration of 2 µg/g BW, BaP significantly suppressed both T- and B-lymphocyte proliferation of medaka splenocytes. In support of these findings, a single IP injection in $\text{B}_6\text{C}_3\text{F}_1$ mice, albeit at a 100-fold higher BaP concentration (200 µg BaP/g BW), also suppressed Con A-stimulated T-cell proliferation (Ginsberg et al., 1989). Reduced T-cell proliferation in response to phytohemagglutinin (PHA) was also observed in $\text{B}_6\text{C}_3\text{F}_1$ mice following 14 daily subcutaneous injections of BaP (yielding a final BaP dose of 200-400 µg/g BW); suppressed LPS-stimulated B-cell proliferation was also observed in this study, but only in those mice receiving the highest cumulative BaP concentration (Dean et al., 1983). Further support for the findings in medaka are provided by *in vitro* studies using anterior kidney lymphocytes collected from spot (*Leiostomus xanthurus*). In that study, a 30 min *in vitro* exposure of kidney cells to a BaP concentration as low as 2nM suppressed (compared to the solvent control) Con A-stimulated T-cell proliferation

(Faisal and Huggett, 1993). In addition, exposure to the 7,8 dihydrodiol metabolite of BaP (BD) also suppressed spot T-cell mitogenesis; co-treatment of cells with ANF and BaP/BD inhibited both the BaP- and BD-induced suppression of mitogenesis.

Findings from the spot *in vitro* study suggested that inhibition of either AhR signal transduction or CYP1A-mediated metabolism of BaP/BD can ameliorate BaP-induced immunosuppression. Thus, splenic lymphocytes from medaka appear to be highly sensitive to the immunotoxic effects of BaP, with suppression occurring at BaP doses 100-fold lower than those previously-observed to affect lymphoproliferative responses in rodents.

Antibody-forming cell numbers were also suppressed in medaka by BaP exposure. Similar BaP-induced reductions in T-dependent PFC number have also been observed by investigators using mammalian systems (White et al., 1994), as well as in a single fish species (Smith et al., 1999). A considerable number of studies have been conducted using rodent models in an attempt to understand the mechanism(s) underlying BaP-induced humoral immune suppression. Results from such studies have demonstrated that BaP-induced reduction of PFC numbers appears to be specific only for T-cell dependent antibody formation, since T-cell independent (polyclonal) responses appear unaffected (Dean et al., 1983; White and Holsapple, 1984; Ladics et al., 1992). Additionally, studies using murine cells revealed that *in vitro* exposure to BaP, or its metabolites BD and BPDE, suppressed the PFC response *in vitro* (Kawabata and White; 1987; 1989), indicating the possible involvement of BaP metabolites in the observed immunosuppression. In other *in vitro* studies, the addition

of ANF, an antagonist of the AhR pathway and CYP1A1 metabolism, abolished BaP-induced suppression of PFC number (Kawabata and White, 1987; 1989; Ladics et al., 1991). This latter finding suggests that either BaP-induced AhR signal transduction or CYP1A-mediated metabolism of BaP may be prerequisites for BaP-induced suppression of the PFC response, at least in rodents.

Finally, a single administration of BaP at 20 or 200 $\mu\text{g/g}$ BW significantly increased host susceptibility to infection with the bacterial fish pathogen *Yersinia ruckeri*. Although BaP has not been previously shown in fish or mammals to reduce host resistance against infection, laboratory exposure of chinook salmon (*Oncorhynchus tshawytscha*) to another PAH, 7,12-dimethylbenz[a]anthracene (DMBA), decreased host resistance against subsequent challenge with the marine bacterial pathogen *Vibrio anguillarum* (Arkoosh et al., 1998). Effects upon medaka host resistance appears to conflict with findings in mammals which demonstrated that exposure to BaP had no effect upon the resistance of exposed mice against infection with *Listeria monocytogenes* (Dean et al., 1983; Ward et al., 1985). The discrepancy between results observed in medaka and those in mice may be due to differences in bacterial pathogenesis. While *L. monocytogenes* infection appears to rely primarily upon evasion of cell-mediated immunity (Bradley, 1995), a compartment of the immune system not highly sensitive to BaP-induced immunotoxicity (Dean et al., 1983), humoral immune defense mechanisms appear to play an important role in mediating *Yersinia* infection by producing antibodies against *Y. ruckeri* necessary for the opsonization and ultimate phagocytosis of the bacterium (Griffin, 1983; Cossarini-

Dunier, 1986; Anderson et al., 1991; Olesen, 1991; Siwicki and Dunier, 1993). Since previously-observed results in rodents (White et al., 1994) and those observed herein have demonstrated the ability of BaP to suppress humoral immunity, perhaps an infectious agent whose clearance/killing is mediated by humoral immune defenses is a more appropriate challenge organism for demonstrating the effects of BaP upon host resistance.

Taken together, results from this study have shown that a single BaP injection can significantly compromise immune function and host resistance in Japanese medaka. Moreover, suppression of phagocyte respiratory burst, lymphocyte proliferation, and humoral immunity were all produced at concentrations of BaP that had no effect upon host survival or which produced obvious changes in overall fish health, indicating the sensitivity of this immune response to BaP exposure. In addition, since BaP exposure of both rodents and medaka results in reduced PFC numbers, it seems possible that BaP may suppress humoral immunity through similar mechanisms in both species.

B. Exposure of Medaka to BaP Results in Induction of CYP1A Expression and Activity Within Immune Organs and Specific Immune Cell Types

CYP1A1-mediated metabolism of BaP has been implicated in its carcinogenic, mutagenic, and immunotoxic properties. Structure-activity relationship (SAR) and AhR antagonism /CYP1A1 inhibition studies using mammalian systems have indicated that BPDE is an ultimate immunotoxic BaP metabolite. Furthermore, studies

in rodent models have demonstrated that lymphoid organs possess both basal and inducible levels of BaP-metabolic activity (White et al., 1994). Both immune (i.e., lymphocytes and macrophages) and non-immune (i.e., bone marrow and thymic stromal cells) cell types within rodent lymphoid tissues demonstrate AhR expression, BaP-mediated CYP1A1 induction, and BPDE production (Ladics et al., 1992a; 1992b; 1992c). Thus, the current hypothesis regarding mammalian BaP immunotoxicity is that immunotoxic BaP metabolites are produced directly (*in situ*) within the immune organs in which, in turn, they evince their effect.

Since there are few reports of the BaP metabolic activity within fish immune cells, this study sought to determine whether CYP1A expression /activity are induced in medaka immune organs /specific cell types following IP injection of BaP. For comparative reasons, CYP1A expression /activity was also monitored in the liver, an organ of high metabolic activity.

Significant induction of CYP1A expression and activity was observed in the liver following exposure of medaka to the two high doses of BaP. Both basal and induced expression of hepatic CYP1A protein appeared within hepatocytes, as determined by IHC. The induction of hepatic CYP1A following BaP exposure (via various routes of administration) has been described for many fish species (Stegeman and Hahn, 1994). The dose-response of BaP-induced hepatic CYP1A is extremely species-dependent and wide variations in the magnitude of induced EROD activities have been demonstrated due to slight differences in assay protocols. Such variation is apparent in this study between EROD activities of liver samples prepared from either

flash-frozen or fresh tissue; however, a similar trend was observed in the BaP-induced EROD activities of both preparations.

In contrast to the liver, splenic microsomes of medaka failed to exhibit any BaP-inducible levels of CYP1A expression or activity. Immunohistochemical examination of spleens revealed that CYP1A expression was only within the endothelial cells of ellipsoids and arteries from medaka exposed to the highest BaP concentration. Low or non-existent splenic CYP1A expression has been previously reported for many fish species (Stegeman and Hahn, 1994). In addition, CYP1A expression has not been observed in selected immune cell types within the teleost spleen following AhR ligand exposure (Stegeman and Hahn, 1994). Lemaire et al. (1990) calculated the splenic half-life of BaP in sea bass following IP injection as 4.5 days compared to 2.2 and 2.4 days for the liver and whole body, respectively. In the same studies, the majority of BaP found in the spleen remained unmetabolized for 17 days, even though the spleen received approximately 3% of the total injected dose. Thus, it seems that the spleen of most fish species exhibits a limited ability to metabolize BaP.

The response of medaka spleen to BaP exposure contrasts with reports of basal and BaP-inducible levels of CYP1A activity in rodent spleen (Ladics et al., 1992a; 1992b; 1992c). The cell type within the rodent spleen most capable of metabolizing BaP is the macrophage, although metabolic activity is also present in splenic lymphocytes. Teleost and mammalian spleens appear to differ somewhat in morphology. Fish spleen lacks the well-developed germinal centers seen in mammals

that are involved in the capture of circulating antigen and subsequent initiation of a humoral response. Although the existence of splenic PFCs following immunization with T-cell dependent antigen has been previously described in other fish species (Kaattari and Arkoosh, 1994), the medaka spleen appears to lack such a capability (Zelikoff et al., unpublished data). The failure of medaka to produce splenic PFCs may be due to the presence of inadequate numbers of macrophages for support of a robust PFC response in this organ (Twerdok et al., 1994).

The observation in Specific Aim 1 of significantly reduced splenic B- and T-lymphocyte proliferation following exposure to BaP, at doses which fail to induce splenic CYP1A activity, does not support the hypothesis that BaP-induced immunotoxicity requires CYP1A-mediated metabolism of BaP. However, studies using rodent lymphocytes have demonstrated up-regulation of CYP1A expression following mitogen exposure due to increased AhR activation in the absence of exogenous ligand (Marcus et al., 1998). Furthermore, induction of CYP1A in rodent lymphocytes by AhR ligands has been demonstrated to depend upon the activation state of the cell, with little TCDD-mediated AhR activation occurring in resting lymphocytes (Lawrence et al., 1996). If considerable amounts of unmetabolized BaP accumulate within the cellular membranes of medaka splenic lymphocytes, mitogen-induced up-regulation of CYP1A expression during the lymphoproliferative response may result in significant metabolism of lymphocyte-associated BaP into reactive immunotoxic metabolites *ex vivo*. In order to test this hypothesis, BaP-exposed medaka lymphocytes were stained for CYP1A protein 24 h following *in vitro* mitogen

exposure. Significantly greater amounts of CYP1A protein were detected within splenocytes incubated with either Con A or LPS compared to unstimulated cells, irrespective of BaP exposure.

In contrast to the spleen, significant induction of kidney microsomal CYP1A expression /activity was observed following exposure of medaka to the highest BaP concentration; basal and BaP-induced kidney CYP1A activity has been previously described in other fish species (Stegeman and Hahn, 1994). The observation that CYP1A expression occurs within the renal tubules following BaP exposure of medaka concurs with previous IHC analyses in other fish species (Stegeman and Hahn, 1994). Unlike the rodent kidney, teleost kidneys serve as both a primary and secondary lymphoid organs. Thus, the immune cells used to determine PFC numbers in medaka were in close proximity to renal tubules which express high levels of BaP-inducible CYP1A activity.

Until recently, the observation of CYP1A expression within mononuclear cells of kidney hematopoietic tissue had not been described for any fish species (Stegeman and Hahn, 1994). In agreement with the current studies, Grinwis et al. (2000; 2001) demonstrated induction of CYP1A expression in a distinct sub-population of kidney mononuclear cells following oral exposure of European flounder (*Platichthys flesus*) to PCB-126 and TCDD. Isolation of kidney mononuclear cells in the current study revealed significantly higher levels of CYP1A protein /activity in non-adherent cells from fish exposed to the highest BaP concentration (compared to vehicle control-exposed fish). Isolated non-adherent cells lacked non-specific esterase activity,

suggesting that these cells were lymphocytes. Glass adherent cells isolated from medaka exposed to the highest BaP dose exhibited increased CYP1A protein expression (compared to vehicle), however, CYP1A induction did not lead to a measurable change in EROD activity. Kidney mononuclear cells adhering to glass demonstrated non-specific esterase activity, suggesting a monocyte lineage for this cell population. Thus, it appears that both lymphocyte- and macrophage-like cells isolated from medaka kidney hematopoietic tissue possess BaP-inducible levels of CYP1A. Rodent splenic lymphocytes and macrophages have also been found to possess inducible CYP1A expression and activity (White et al., 1994).

Thus, the studies undertaken to validate Aim 2 determined that exposure of medaka to BaP resulted in the induction of CYP1A expression /activity within the liver and kidney. Furthermore, BaP-induced expression of CYP1A was observed both in the excretory tubules and hematopoietic tissue of the kidney, suggesting that BaP can be metabolized within this immune organ. Isolation of kidney mononuclear cells revealed that both lymphocyte- and macrophage-like cell populations possess BaP-inducible CYP1A protein. Therefore, it appears that BaP could be metabolized directly within medaka immune cells previously demonstrated to be sensitive to the immunomodulatory effects of BaP exposure.

C. Inhibition of CYP1A Activity Results in Reduced Immunotoxicity

Following Acute Exposure to BaP and BD, but not BPDE

1. In Vivo Studies

Numerous rodent studies have indicated the importance of BaP metabolism in its observed immunotoxicity (White et al., 1994). The inability of BeP to alter rodent immune function was demonstrated over 30 years ago (Stjernsward, 1966), and this observation has since been substantiated by more recent investigations employing both *in vivo* and *in vitro* models (Zwilling, 1977; Dean et al., 1983; White and Holsapple, 1984; Urso et al., 1986). Although BeP and BaP are structurally similar, BeP is a very weak AhR ligand and not a CYP1A1 substrate (Bigelow and Nebert, 1982). Furthermore, *in vitro* suppression of PFC numbers induced by BaP is ameliorated by concurrent exposure of rodent splenocytes to the AhR antagonist and CYP1A1 inhibitor, ANF (Kawabata and White, 1989). Although the aforementioned mammalian studies failed to determine whether the ANF dose prevented BaP-induced CYP1A1 protein expression, BaP-induced EROD activity was inhibited at doses of ANF that ameliorated BaP immunotoxicity.

Since there is little information regarding the importance of CYP1A-mediated metabolism of BaP in BaP-induced immunosuppression of fish, this study sought to determine: (1) the effects of BeP exposure upon medaka humoral immunity; and, (2) the effects of inhibition of AhR activation and/or CYP1A-mediated metabolism upon BaP-induced immunosuppression.

Exposure of medaka to BeP at concentrations as high as 400 $\mu\text{g/g}$ BW had no effect upon medaka humoral immunity. This is the first observation that PAH-induced immunosuppression in fish follows a similar SAR as observed in mammals; numerous rodent studies have also failed to observe BeP-induced immunotoxicity (White et al., 1994). In agreement with previous rodent and fish liver cell culture studies (Bols et al., 1999), BeP failed to induce microsomal CYP1A expression / activity in medaka liver and kidney. In contrast to data obtained from microsomes, IHC did reveal a slight induction of CYP1A expression in medaka hepatocytes, kidney hematopoietic tissue, and splenic endothelia following exposure of fish to the two highest concentrations of BeP. This may be due to the fact that BeP does possess some AhR agonist activity, although significantly less than that described for BaP (Bols et al., 1999). For example, BeP was demonstrated to induce EROD activity in a rainbow trout liver cell line with an ED_{50} of 286 nM and maximal EROD activity of 8.7 pmoles /mg /min (Bols et al., 1999). In comparison, the same study demonstrated that BaP possessed an ED_{50} of 28.5 nM for EROD induction, a maximal EROD activity of 39.3 pmoles /mg /min, and a toxic equivalency factor (TEF) ten times greater than that of BeP.

The inability of BeP to induce immunotoxicity in fish suggests a need for AhR activation and/or CYP1A-mediated metabolism in the observed BaP-induced immunosuppression. Since BeP is structurally similar to BaP, it could be postulated that both parent compounds possess the ability to non-specifically interact with biological membranes in an identical manner. Thus, the inability of BeP to induce immunotoxicity provides evidence that BaP probably does not induce immunotoxicity

by a direct mechanism such as altered phospholipid membrane functions.

Furthermore, BeP has been demonstrated to interact with the 4S BaP-binding protein with an affinity equal to that of BaP in rodents (Lesca et al., 1993; Sterling et al., 1994), suggesting a minimal involvement of BaP-4S protein interactions in the observed immunotoxicity.

Concurrent exposure of medaka to ANF and BaP ameliorated the suppression of PFC numbers observed following exposure of fish to BaP alone. The ability of ANF to ameliorate BaP-induced reduction in PFC numbers has also been described in rodents using *in vitro* exposure systems (White et al., 1994). However, this is the first study (in fish or mammals) that describes such an inhibitory effect of ANF upon BaP-induced suppression of the PFC response following *in vivo* exposure. In support of this study, Reynaud et al. (2001) observed that concurrent injection of carp (*Cyprinus carpio*) with ANF and 3-MC reversed immune alterations in macrophage respiratory burst activity produced following exposure to 3-MC alone.

Interestingly, exposure of medaka to ANF alone resulted in significant induction of CYP1A expression within hepatocytes and renal tubules; however, only slight increases in microsomal CYP1A protein levels were observed for liver and kidney. In addition, a significant increase (compared to vehicle control) in CYP1A index was observed in kidney mononuclear cells (both non-adherent and adherent) exposed to ANF. The ability of ANF to induce CYP1A protein expression following *in vivo* exposure has been previously described for fish (Williams et al., 1998) and rodents (Sinal et al., 1999). *In vitro* rodent studies have shown that high concentrations

of ANF can promote AhR activation leading to up-regulation of CYP1A1 expression (Wilhelmsson et al., 1994). Thus, the observed induction of CYP1A expression by ANF in this study may be due to its previously described agonist properties at high concentrations. In contrast to the current findings regarding ANF-induced CYP1A expression, exposure of fish to ANF did not result in elevated EROD activities in liver, kidney, or isolated kidney mononuclear cells.

Concurrent exposure of medaka to ANF and BaP did not appear to antagonize the induction of CYP1A protein within renal and hepatic microsomes, hepatocytes, renal tubules, splenic endothelia, or isolated kidney mononuclear cells. This observation indicates that ANF may not be acting as an AhR antagonist in the medaka system. In contrast, the ANF and BaP combined exposure group failed to exhibit significant induction of EROD activity in liver, kidney, and isolated kidney mononuclear cells. The observation that combined ANF and BaP exposure results in induced levels of CYP1A protein, but not CYP1A activity, suggests that ANF may be acting as a CYP1A inhibitor and not an AhR antagonist *in vivo*; previous *in vitro* rodent studies have demonstrated the ability of ANF to competitively inhibit CYP1A activity (Koley et al., 1997).

Since ANF appears to act as a CYP1A inhibitor in both isolated kidney immune cells and other tissues within the kidney, the reversal of BaP-induced immunosuppression in medaka observed following combined exposure to BaP and ANF may be a result of decreased formation of immunotoxic BaP metabolites within the kidney. CYP1A-independent mechanisms, such as the involvement of AhR battery

genes other than CYP1A and AhR cross talk, are less likely explanations for the observed BaP-induced suppression of PFC numbers, since the AhR pathway appeared to be either activated or unaffected in the kidney by ANF and BaP combined exposure. It is important to note that no reports exist regarding the possible effects of ANF upon AhR-regulated gene products other than CYP1A1. To further investigate the involvement of CYP1A-mediated metabolism of BaP in the observed immunosuppression, studies were performed using DHEA (a compound previously shown not to affect AhR signaling but to inhibit CYP1A activity *in vitro*; Ciolino and Yeh, 1999).

Exposure of fish to DHEA alone failed to induce CYP1A expression /activity in liver, kidney, and isolated kidney mononuclear cells. In addition, combined exposure of fish to DHEA and BaP resulted in CYP1A expression levels (within the liver, kidney, or isolated kidney mononuclear cells) that were comparable to those observed following exposure to BaP alone. Although DHEA did not interfere with BaP-induced CYP1A expression, combined DHEA and BaP exposure resulted in CYP1A activities comparable to those of vehicle-exposed fish. The inability of DHEA to interfere with BaP-induced CYP1A expression is in accordance with observations of rodent studies in which DHEA failed to interfere with AhR ligand binding and transactivation (Ciolino and Yeh, 1999). However, these same studies demonstrated that DHEA also decreased CYP1A1 protein expression by a post-transcriptional mechanism, which apparently did not occur in the current study since the combined DHEA and BaP exposure group exhibited significantly elevated levels of CYP1A

protein (compared to vehicle control). Thus, the effects of DHEA upon CYP1A expression /activity appear to be similar to those of ANF. However, DHEA treatment alone does not alter CYP1A expression as observed following ANF exposure.

Medaka exposed to DHEA and BaP concurrently exhibited a reversal of BaP-induced immunosuppression similar to that observed following combined exposure of fish to ANF and BaP. Thus, from inhibitor studies employing both ANF and DHEA, it is possible that BaP-induced suppression of PFC numbers may require CYP1A-mediated metabolism of BaP. Since isolated kidney immune cells possessed basal and BaP-inducible levels of CYP1A activity, it is possible that these cells can produce immunotoxic BaP metabolites *in situ*. Furthermore, renal tubules were found to exhibit similar metabolic properties to those determined for kidney mononuclear cells. It is likely that CYP1A-mediated metabolism of BaP within this tissue may also contribute to the observed immunotoxicity by providing immunotoxic BaP metabolites to the surrounding kidney immune cells. Finally, rodent studies have determined that immunotoxic BaP metabolites can be transported in the bloodstream to sites distant from where they were produced (Ginsberg and Atherholt, 1989; 1990). Since the medaka liver possesses high metabolic activity, BaP metabolites produced within this organ could be transported to the kidney and subsequently alter immune function. All three of the aforementioned sources of BaP metabolites could potentially be involved in BaP-induced immunosuppression in medaka. The demonstration of immunosuppression following *in vitro* exposure of medaka immune cells to BaP or

BaP metabolites provides evidence that kidney immune cells are capable of forming immunotoxic BaP metabolites *in situ*.

2. *In Vitro* Studies

It has been previously demonstrated that rodent immune cells are sensitive to direct (*in vitro*) exposure to BaP (White et al., 1994). Furthermore, *in vitro* exposure of rodent splenocytes to BaP metabolites (i.e., BD or BPDE) also results in reduced PFC numbers. Altered immune function observed following *in vitro* exposure to BaP or BD can be reversed by co-incubation of cells with ANF (Kawabata and White, 1987; 1989). The aforementioned studies indicate that rodent immune cells are also capable of directly metabolizing BaP into reactive immunotoxic metabolites.

Since little is known concerning BaP sensitivity and the metabolic capability of fish immune cells, this Specific Aim sought to determine whether medaka kidney cells were sensitive to BaP-, BD-, or BPDE-induced immunomodulation. Furthermore, the effects of AhR antagonism / CYP1A inhibition upon BaP /BaP metabolite-induced immunosuppression *in vitro* were investigated.

Exposure of isolated kidney mononuclear cells to BaP, BD, and BPDE resulted in significant suppression of PFC numbers. As stated previously, a similar suppression of immune function following exposure to BaP, BD, and BPDE has been described for rodents (White et al., 1994). Furthermore, previous rodent studies have demonstrated that BaP, BD, and BPDE all exhibit comparable immunotoxic potencies (Kawabata and White, 1987; 1989). Although it would be expected that either BD or BPDE

would induce greater immunosuppression than non-metabolized BaP, these metabolites may not be taken up into immune cells as readily as BaP. In addition, both BD and BPDE may become inactivated by reacting with media components (i.e., serum) outside the cell or by rapid conjugation to sulfates or glucuronides within the cell (i.e., phase II biotransformation).

The ability of BaP and BD to directly alter immune function suggests that medaka immune cells are capable of metabolizing BaP and BD into reactive metabolites. Analysis of CYP1A expression /activity within immune cells following BaP exposure revealed that immunotoxic doses of BaP can significantly induce CYP1A expression and activity within both adherent (i.e., macrophage-like) and non-adherent (i.e., lymphocyte-like) cell populations. It has been previously demonstrated that rodent splenocytes (both macrophages and lymphocytes) possess both basal and BaP-induced levels of CYP1A activity (Ladics et al., 1992a; 1992b; 1992c). The ability of BD to induce CYP1A expression /activity in this study appeared to be limited to non-adherent (i.e., lymphocyte-like) cells; possibly corresponding to the slightly reduced ability of BD to induce immunosuppression as compared to BaP. Previous studies using rodent liver cells have demonstrated that BD is a very weak agonist of the AhR, resulting in poor induction of EROD activity (Bigelow and Nebert, 1982). However, studies using rodent splenocytes have determined that the basal metabolic activity of macrophages is sufficient for the production of BPDE from BD (Ladics et al., 1992b). Furthermore, the comparable immunotoxicities of BaP and BD observed in this study may be due to the reduced capability of BD to induce

CYP1A expression /activity compared to BaP. Exposure of immune cells to BPDE in the current study did not induce CYP1A expression /activity in either immune cell population; suggesting no relationship between BPDE immunotoxicity and metabolism (CYP1A-independent). Data from rodent studies demonstrate that BPDE has little AhR agonist activity (Bigelow and Nebert, 1982). In fact, 0.01 and 1.0 μ M BPDE significantly suppressed adherent cell EROD activity without affecting CYP1A protein levels in the current study. The observed CYP1A-inactivation may be the result of BPDE-induced oxidative stress, since studies in scup have revealed that teleost CYP1A is highly susceptible to PCB-induced oxidative inactivation (Schlezingner and Stegeman, 2001).

BaP, BD, and BPDE all appear capable of suppressing the immune function of medaka cells. The immunotoxicity of BaP and BD was demonstrated to correlate with induction of CYP1A expression /activity within medaka immune cells. In contrast, BPDE was observed to suppress immune function without altering CYP1A expression. Previous rodent studies have demonstrated the amelioration of BaP- and BD-induced suppression of PFC numbers following co-incubation with ANF. This finding suggests that further metabolism of these compounds is required for induction of immunotoxicity. Although *in vitro* studies using rodent and human lymphocytes have demonstrated the inability of ANF to alleviate BPDE-induced immunosuppression (Davila et al., 1996; Mounho and Burchiel, 1998), ANF /BPDE co-incubation has not been applied to studies investigating the PFC response. Furthermore, rodent studies employing ANF and BaP only investigated BaP-

metabolism and did not determine whether the doses of ANF utilized in the study inhibited CYP1A expression (Kawabata and White, 1989). Thus, studies in this Aim employed the use of AhR antagonist /CYP1A inhibitors to investigate the role(s) that AhR activation and/or CYP1A activity play in BaP-, BD-, and BPDE-induced immunosuppression in medaka.

Concurrent exposure of medaka immune cells to MNF and BaP, BD, or BPDE failed to reverse the reduction in PFC numbers observed following exposure to the immunotoxic compounds alone. MNF has been previously demonstrated to act as a pure AhR antagonist *in vitro* with little effects upon CYP1A activity (Dertinger et al., 2000). In the current study, not only was MNF observed to be slightly immunotoxic on its own, but MNF exposure resulted in significant induction of CYP1A expression and activity within medaka immune cells. Furthermore, combined exposure of medaka cells to MNF and BaP /BD failed to inhibit BaP- or BD-induced CYP1A expression or activity. It seems apparent that, in medaka immune cells, MNF acts as an AhR agonist and does not inhibit CYP1A activity. Previous studies investigating the effects of MNF upon rainbow trout CYP1A expression also demonstrated an AhR agonist property for this compound in fish (Dr. T. Gasiewicz, personal communication).

Combined exposure of medaka immune cells to ANF and either BaP, BD, or BPDE demonstrated elimination of BaP- and BD-induced immunosuppression, but not BPDE-induced reduction in PFC numbers. In addition, ANF appeared to operate as an AhR antagonist in this study because combined exposure of medaka immune cells to ANF and either BaP or BD inhibited induction of CYP1A expression /activity. Unlike

the *in vivo* effects of ANF in medaka, *in vitro* exposure to ANF alone did not induce CYP1A expression. The ability of ANF to act as an AhR antagonist has been described previously in *in vitro* rodent studies (Lu et al., 1996; Henry et al., 1999). Therefore, both BaP and BD appear to require further metabolism to produce immunotoxicity. The immunotoxicity induced by BPDE apparently does not rely upon either AhR activation or CYP1A-mediated metabolism.

Similar to that observed following MNF exposure, DHEA failed to reverse BaP-, BD-, or BPDE-induced immunosuppression in co-incubation studies. In fact, exposure to DHEA alone resulted in a slight (non-significant) reduction in PFC numbers. Furthermore, concurrent *in vitro* exposure of immune cells to DHEA and either BaP or BD resulted in significant inhibition of both CYP1A expression and EROD activity. These aforementioned results contrast somewhat from that observed following *in vivo* DHEA exposure; co-incubation of immune cells with DHEA and BaP inhibited BaP-induced CYP1A expression, whereas *in vivo* co-injection of DHEA and BaP failed to alter this particular endpoint. In the *in vitro* studies, DHEA appears to be acting as an AhR antagonist. However, DHEA may also affect fish immune cells in a manner similar to that described in rodent cells, as a post-transcriptional inhibitor of CYP1A expression (Ciolino and Yeh, 1999). The observation that DHEA failed to reverse BaP- and BD-induced immunosuppression, yet inhibited induction of CYP1A expression /activity, is difficult to interpret. Perhaps the slight reduction in PFC numbers observed following exposure of cells to DHEA alone demonstrates the potential of this compound to act as an immunotoxicant *in vitro*. Furthermore,

combined exposure of cells to DHEA and either BaP or BD only exacerbates DHEA-induced immunomodulation.

Similar to that seen in the ANF studies, concurrent treatment of medaka cells to ELP and either BaP, BD, or BPDE reversed both BaP- and BD-induced immunosuppression, but not BPDE-induced reduction of PFC numbers. Combined exposure of immune cells to ELP and BaP or BD did not inhibit BaP- or BD-induced CYP1A expression. Furthermore, exposure of immune cells to ELP alone resulted in significant induction of CYP1A protein levels within both adherent and non-adherent cell populations. Thus, it appears that ELP is acting as an AhR agonist in these studies. In contrast, concurrent exposure to ELP and either BaP or BD resulted in inhibition of BaP- and BD-induced EROD activity within immune cells. Previous studies utilizing rodent cells have demonstrated that ELP possesses a potent CYP1A1 inhibitory activity (Zhao and Ramos, 1995; Bowes et al., 1996). In the current study ELP also appears to exhibit AhR agonist and CYP1A inhibitor properties. The ability of ELP to reverse the immunotoxicity of both BaP and BD demonstrates the importance of CYP1A-mediated metabolism in immunosuppression. Since ELP does not appear to interfere with BaP- or BD-induced AhR signaling, it could be suggested that the resulting immunotoxicity does not rely upon CYP1A-independent mechanisms, such as either up-regulation of AhR battery genes other than CYP1A or AhR cross talk. In contrast, BPDE-induced immunosuppression is independent of CYP1A activity, indicating that this BaP metabolite may be an ultimate immunotoxic compound in medaka.

VII. CONCLUSIONS

The results obtained from these studies clearly demonstrate the potential of BaP exposure to alter the immune response of medaka. Significant suppression of medaka innate and adaptive immunity were observed following exposure of fish to BaP doses that failed to affect host survival or general health indices. Furthermore, increased host susceptibility to bacterial infection was demonstrated in fish exposed to BaP doses previously shown to alter host immunocompetence. Of greatest interest was the observation that medaka T-lymphocyte-dependent antibody production was significantly suppressed following BaP exposure, thought to be the hallmark of BaP-induced immunosuppression in rodent models (reviewed by White et al., 1994).

Since it has been demonstrated in rodent studies that BaP-induced immunosuppression is likely dependent upon the metabolism of BaP into immunotoxic metabolites (White et al., 1994), levels of CYP1A expression and activity within medaka immune organs were determined. Exposure of medaka to BaP resulted in significant induction of CYP1A expression and activity within kidney tubules and residing mononuclear cells. Isolated kidney mononuclear cells exposed to BaP *in vivo* revealed that both macrophage and lymphocyte cell populations possessed CYP1A activity. Therefore, BaP can be metabolized directly within medaka kidney, either by adjacent renal tubules or within mononuclear cells of the hematopoietic tissue.

A structure-activity relationship was determined for BaP-induced suppression of PFC numbers by the utilization of the BaP congener, BeP. Exposure of medaka to BeP failed to affect humoral immunity, suggesting that the ability of BaP to alter immune function relies upon either its activity as an AhR agonist or its metabolism into reactive metabolites.

Further studies assessing the importance of BaP metabolism in the observed immunotoxicity examined the effects of CYP1A inhibitors. Exposure of medaka to ANF in combination with BaP reversed BaP-induced suppression of PFC numbers. Moreover, ANF exposure inhibited BaP-induced EROD activity within the kidney and isolated mononuclear cells. In addition, DHEA produced effects similar to those of ANF on BaP-induced immunosuppression and CYP1A activity. These *in vivo* inhibitor studies provide strong evidence for the importance of CYP1A-mediated metabolism of BaP in producing suppression of PFC numbers in medaka. Events such as AhR up-regulation of genes other than CYP1A or AhR cross talk are unlikely to significantly contribute towards BaP-induced suppression of humoral immunity since ANF and DHEA did not interfere with the induction of CYP1A protein.

Medaka immune cells were also exposed *in vitro* to BaP or BaP metabolites so as to assess the ability of these compounds to directly alter the function of immune cells. Significant suppression of PFC numbers was demonstrated following exposure of medaka immune cells to BaP, BD, or BPDE. Both BaP and BD, but not BPDE, were capable of inducing CYP1A expression /activity within immune cells. Furthermore, concurrent exposure of medaka cells to AhR antagonists /CYP1A

inhibitors and BaP or BD reversed both BaP- and BD-induced suppression of PFC numbers; concurrent exposure of cells to AhR antagonists /CYP1A inhibitors and BPDE failed to alter BPDE-induced immunosuppression. Thus, CYP1A-mediated metabolism within medaka immune cells appears important for immunosuppression induced by BaP and BD, but not for BPDE. In agreement with previous rodent studies, both BaP and BD require metabolism into more reactive BaP metabolites prior to inducing immunosuppression. BPDE may, indeed, represent an ultimate immunotoxic metabolite since BPDE-induced immunosuppression appears to be unaffected by inhibition of CYP1A activity.

This study provides evidence that BaP-induced immunotoxicity in both fish and rodents occur through similar cellular mechanisms. The demonstration that CYP1A-mediated metabolism may be required for BaP-induced immunotoxicity in medaka is in agreement with previous rodent studies. Therefore, this study provides clear evidence that the medaka model is sensitive to a known mammalian immunotoxicant, BaP. Further studies utilizing medaka must employ additional mammalian immunosuppressants, such as cyclophosphamide and cyclosporin A, in order to fully substantiate the use of medaka in immunotoxicological studies.

Increased formation and environmental release of BaP (and other PAHs) is likely to occur near areas of high anthropogenic activity. Aquatic ecosystems have been documented to possess extremely high levels of BaP. Thus, the potential for exposure of feral fish populations to BaP is evident. Furthermore, feral fish may also be exposed to various other PAHs (at even higher concentrations than BaP) that may

exhibit similar properties to those of BaP. Future research is needed regarding the immunotoxic potential of other environmentally relevant PAHs, or PAH mixtures, in order to better assess the risk of PAH exposure upon aquatic ecosystems. Determination of the mechanisms of action for such compounds may allow researchers to more quickly identify potential immunotoxicants.

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