

THE DEVELOPMENT AND APPLICATION OF A HEMOLYTIC PLAQUE
FORMING CELL ASSAY (PFC) AND A CYTOTOXIC T-LYMPHOCYTE ASSAY
(CTL) IN TILAPIA (*Oreochromis niloticus*) FOR IMMUNOTOXICITY RISK
ASSESSMENT OF ENVIRONMENTAL CONTAMINANTS

by

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The Development and Application of a Hemolytic Plaque Forming Cell Assay (PFC) and a Cytotoxic T-Lymphocyte Assay (CTL) in Tilapia (*Oreochromis niloticus*) for Immunotoxicity Risk Assessment of Environmental Contaminants

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(ABSTRACT)

The prospect of utilizing the cichlid teleost tilapia (*Oreochromis niloticus*) as an alternative experimental model to mammals for immunotoxicity risk assessment is currently being proposed. As such, the National Toxicology Program's (NTP) standard battery of rodent immunotoxicity assays is being developed for use in this fish species. Included in the testing series are the hemolytic plaque forming cell (PFC) and the cytotoxic T-lymphocyte (CTL) assays, quantitative indicators of antibody production and cell-mediated activity, respectively. The assays were modified in consideration of specific tilapian immune parameters, then tested using fourteen environmental contaminants or drugs, ten of which are classified by the NTP as immunotoxic in rodents. Reduced antibody production via a decrease in plaque number was observed in response to exposure of tilapia to eight of the nine humoral immunotoxicants, and five of the five non-immunotoxicants. Under specific immunization circumstances, immunostimulation (also a response to

immunotoxicity) was noted via an increase in plaque number in benzo[a]pyrene (B[a]P) exposed fish using the PFC assay, a result noted in rodents as well. Reduced T-cell recognition and lysis of allogeneic tilapia lymphocytes via a decrease in the percentage of specific ⁵¹Chromium (⁵¹Cr) release was observed in response to exposure of tilapia to the nine of the ten cell-mediated immunotoxicants, and four of the four non-immunotoxicants. Although the normal teleost immune responsiveness was slightly weaker than seen with mice under comparable conditions (presumably due to differences in antibody structure and decreased cells counts), tilapia were found to exhibit well-defined humoral and cell-mediated immune responses, and responses to immunotoxic and non-immunotoxic chemicals comparable to the rodent model.

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Table of Contents

	<u>Page</u>
Abstract	ii
Acknowledgements	iv
List of Figures	vii
INTRODUCTION	1
LITERATURE REVIEW	4
A. Comparative Immunology of the Fish and Rodent	4
1. Non-specific Immunity	5
2. Specific Immunity	8
a. Humoral Immunity	9
b. Cell-Mediated Immunity.....	13
B. The National Toxicology Program's Development of Immunotoxicity Risk Assessment Standards	16
1. Hemolytic Plaque Forming Assay (PFC)	18
2. Cytotoxic T-Lymphocyte Assay (CTL)	19
C. Environmental Contaminants, Chemicals and Toxins	20
1. Review of Selected Immunotoxicants for Study.....	20
a. Azathioprine (AZA).....	20
b. Polycyclic Aromatic Hydrocarbons: Benzo[a]pyrene (B[a]P) and 7,12-Dimethylbenzanthracene (DMBA)	21
c. Cadmium chloride (CdCl ₂)	23
d. Diethylstilbesterol (DES)	24
e. N-nitrosodimethylamine (DMN)	24
f. Lindane (HCH γ)	25
g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	26
h. T ₂ -Mycotoxin (T ₂ -toxin).....	28
2. Review of Selected Non-immunotoxicants for Study	30
a. Acetonitrile (Methyl cyanide).....	30
b. Formaldehyde	30
c. Oxymethalone	30
d. Toluene	31
e. Tert-butylhydroquinone (TBHQ).....	32
LIST OF REFERENCES	34

MANUSCRIPT I. THE HEMOLYTIC PLAQUE FORMING CELL ASSAY (PFC) IN TILAPIA (<i>Oreochromis niloticus</i>) EXPOSED TO BENZO[a]PYRENE: ENHANCED OR DEPRESSED PLAQUE FORMATION DEPENDS ON DOSING SCHEDULE	55
Abstract	55
Introduction.....	56
Materials and Methods.....	59
Results	66
Discussion	68
Acknowledgements	73
References.....	74
Tables and Figures	81
 MANUSCRIPT II. TILAPIA (<i>Oreochromis niloticus</i>) AND RODENTS EXHIBIT SIMILAR IMMUNOTOXICITY RISK ASSESSMENT RESULTS USING A MODIFIED HEMOLYTIC PLAQUE FORMING CELL (PFC) ASSAY	88
Abstract	88
Introduction.....	89
Materials and Methods.....	91
Results	97
Discussion	98
Acknowledgements	103
References	104
Tables and Figures	108
 MANUSCRIPT III. THE DEVELOPMENT AND APPLICATION OF A CYTOTOXIC T-LYMPHOCYTE ASSAY (CTL) IN TILAPIA (<i>Oreochromis niloticus</i>) FOR IMMUNOTOXICITY RISK ASSESSMENT YIELDS RESULTS SIMILAR TO THAT OF THE RODENT MODEL	130
Abstract	130
Introduction.....	131
Materials and Methods.....	134
Results	142
Discussion	144
Acknowledgements	148
References.....	149
Tables and Figures	154
 SUMMARY.....	174
 VITAE	178

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1.1 Schematic model of the major type of fish immunoglobulin in its tetrametric form	44
1.2 The National Toxicology Program's panel for detecting immune alterations following chemical or drug exposure in rodents	45
1.3 The National Toxicology Program's immune panel assessing individual and pairwise concordance establishing predictability of immunosuppression	46
1.4 Plaque formation in SRBC agar	47
1.5 Structure of Azathioprine (Aza)	47
1.6 Structure of Benzo[a]Pyrene (B[a]P)	48
1.7 Structure of 7-12-dimethylbenz(a)anthracene (DMBA)	48
1.8 Structure of Diethylstilbesterol (DES)	49
1.9 Structure of N-nitrosodimethylamine (DMN)	49
1.10 Structure of Lindane (<i>g</i> -HCH)	50
1.11 Structure of 2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin (TCDD)	50
1.12 Proposed Toxic Mechanism of TCDD	51
1.13 Structure of T ₂ Mycotoxin (T ₂)	51
1.14 Structure of Acetonitrile	52
1.15 Structure of Formaldehyde	52
1.16 Structure of Oxymethalone	53
1.17 Structure of Toluene	53

INTRODUCTION

Recent documents prepared by the National Research Council (1) and the Office of Technology Assessment (2) focusing on immunotoxicity have cited growing concern within the scientific and public communities regarding the prevalence of toxic agents in the environment and the subsequent increase of immune function impairment and disease. Additionally, the political climate of the last decade concerning the use of mammalian models in toxicity research has promoted the development of assays in alternative, non-mammalian species. As such, we have endeavored to comprehensively examine the development of the National Toxicology Program's (NTP) standard battery of immunotoxic assays (3-4) in the teleost tilapia, and compare the predictive values of immunosuppression obtained to the standard B6C3F1 mouse model.

The rationales behind the selection of a fish model for toxic risk assessment include the fact that fish provide an exposure model relevant to natural settings where they are bathed continuously in polluted aquatic environments (5-6). Moreover, it has been confirmed by numerous studies that fish have immune processes and metabolic capacities comparable to mammals, possessing similar critical biochemical pathways involved in xenobiotic chemical conjugation, detoxification and activation, and DNA adduct formation and repair (6-9). As an example, cytochrome P450 liver microsome induction by polycyclic aromatic hydrocarbons (PAHs) has been demonstrated in tilapia

hybrids and other fish species (10-12). These studies and others have promoted the scientific acceptance of fish as viable research models.

Tilapia were identified as a potential fish model for the present series of chemical immunotoxicity studies for several reasons. First, the aquaculture of tilapia as a food source is ranked second and fourth for the world and the United States, respectively (13). As it is common aquaculture practice to use water derived by possibly contaminated natural sources (5, 13), assessment of the specific immunotoxic responses in tilapia has worldwide health and economic importance. Second, at an average adult weight of 100-250+g, tilapia offer the advantages of larger tissue mass and greater lymphocyte numbers as compared to the other smaller fish models used in toxicity testing, such as the fathead minnow (*Pimephales promelas*) and medaka (*Oryzias latipes*). Third, tilapia are easily bred and maintained in the laboratory setting. Finally, tilapia are inexpensive, currently costing approximately \$0.75 each compared to an average cost of approximately \$13.00 each for B6C3F1 mice.

It is well documented that in mammals and fish, sub-lethal exposure to certain environmental contaminants, as well as certain drugs and toxins, may selectively target the immune system. In such cases, aberrations in the immune system may occur at concentrations well below those that produce overt gross morphological abnormalities or death. Therefore, the immune system is an important organ system when considering the toxicant-induced effects of chemical exposure (5-6, 13-15).

This research project focuses on the tilapian requirements for the development and implementation of two important tests of the NTP: The hemolytic plaque forming cell assay (PFC) to assess humoral immunity, and the cytotoxic T-lymphocyte assay (CTL), denoting cell-mediated immunity. When performed individually, estimated predictive values of the PFC and CTL assays for actual immunosuppression in B6C3F1 mice are 78% and 67%, respectively. When performed concurrently, to date all chemicals tested that inhibit both the PFC and CTL assays also produce significant immunosuppression (thus the pairwise predictive value of these tests is 100%). For this reason, these two assays are among the most potent indicators available for immunotoxicity risk assessment (3-4).

Following development, the newly standardized assays were evaluated in tilapia exposed to fourteen environmental contaminants, drugs, or toxins. Of these, nine are known immunosuppressive compounds in the rodent model, and include azathioprine, benzo[a]pyrene (B[a]P), cadmium chloride (CdCl₂), diethylstilbesterol (DES), 7,12-dimethylbenzanthracene (DMBA), dimethylnitrosamine (DMN), hexachlorocyclohexane (lindane), T₂ mycotoxin (T₂-toxin), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Five are classified as non-immunosuppressive, and include acetonitrile, formaldehyde, oxymethalone, T-butylhydroquinone, and toluene (however, toluene has subsequently been found to be immunotoxic in rodents and other species). These data were then compared to the standard rodent model.

Our data indicate that tilapia respond in a manner similar to laboratory rodents when challenged with known immunotoxic and non-immunotoxic chemicals, thus fish may have the potential for development as an alternate species to mammals for use in preliminary chemical immunotoxicant detection and immunotoxicity assessment.

LITERATURE REVIEW

A. Comparative Immunology of Fish and Mammals

Of the greater than 20,000 known species of fish, comparatively few have been studied in a research setting; less still immunologically (13-15). However, of the main species examined, including the fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), and carp (*Cyprinus carpio*), it is clear that teleost fish and mammals exhibit much immunological similarity (13-17). While important phenotypic and mechanistic differences do exist, these do not seem to curtail the efficacy of specific and non-specific immune comparison assays between the mammalian and teleost species (18-22).

The immune system is primarily concerned with resistance against foreign invaders and protection against neoplastic cells. Leukocytes (B- and T-lymphocytes, natural killer (NK) cells, macrophages and granulocytes) are the major cellular players involved, arising during development from stem cells

within the yolk sac and then the mammalian fetal liver. Immune cells in adult mammals are produced primarily by the bone marrow, which serves both hematopoetic and immunopoetic functions. This organ is of paramount importance, as it is the only lymphoid tissue capable of providing the complete restoration of primary lymphoid tissue (spleen, thymus, lymph nodes and liver), and therefore can alone prevent adult mammal death following lethal irradiation. In most mammalian species, the bone marrow itself acts also as a primary and secondary lymphoid organ, regulating the production, differentiation, and maturation of lymphocytes (23-24).

The fish species studied to date do not possess bone marrow, yet lymphoid cells nearly identical in structure and function to mammalian cells are present, arising from stem cells located mainly within the anterior kidney (or pronephros tissue). This small, reddish, paired organ located anteriorly just above the pharyngeal plates (upper mouth cartilage) in tilapia functions similarly to mammalian bone marrow in that it is hematopoetic, immunopoetic, and a primary and secondary lymphoid organ along with the teleost spleen, thymus and liver (teleost lymph nodes are not present) (5, 13-14).

1. Non-Specific Immunity

In fish and mammals, an infectious disease process begins with penetrance of the first defense barriers by foreign invaders. The mammalian physical and

chemical barriers include the skin, mucous membranes, saliva, and gastrointestinal and other secretions, consisting of symbiotic bacteria or lytic enzymes to destroy or halt the progress of the pathogen (23-25).

In fish, the initial barriers are a viscous mucous consisting of lytic enzymes covering the entire animal, a thin layer of living epidermal cells (not dead as in mammals), and the scales. The mucous traps and/or destroys most pathogens, and the scales are almost impenetrable unless the pathogen is capable of lateral movement around the scales. However, fish are more vulnerable in areas where there are no scales present, and since their external epidermal surfaces are composed of living cells, fish live more intimately with their environment than do terrestrial mammals, sometimes leading to easier pathogen invasion (5, 26).

After invader penetration, both fish and mammals exert similar first-line defense immunity, which is non-specific in nature and therefore does not involve prior pathogen sensitization. These reactions primarily involve myeloid and mononuclear phagocytes, which respond to chemotactic factors (produced by the affected tissue cells or the invader itself) and destroy any foreign particles or microorganisms quickly and indiscriminantly via phagocytosis (27).

Phagocytic destruction involves engulfment of the invader into vacuoles and lysosomes, followed by a lethal respiratory burst reaction. This process uses H_2O_2 , myeloperoxidase or catalase, and halide anions to produce

hypohalides, especially OCl_2 , a very powerful reactive oxygen species that rapidly attacks and destroys most biological molecules (28).

The myeloid cells, also called granulocytes or polymorphonuclear leukocytes, are generally the first defenders to arrive at the invasion site. They perform phagocytosis quickly, but are short-lived and therefore cannot mount a sustained reaction. Granules consisting of potent bactericidal enzymes such as myeloperoxidase, defensin and lysozyme are also present, enhancing phagocytic destruction. Myeloid cells possess irregularly shaped, lobulated nuclei, and fall into three types depending on their ability to take up certain dyes: basophils (acidic), eosinophils (basic), and neutrophils (neither acidic or basic). Neutrophils make up 80% of all granulocytes, and are the most important cell in first-line defense reactions (28).

The mononuclear phagocytic cells of mammals are primarily tissue macrophages (also called Kupffer cells in the liver, Langerhan's cells in the skin, microglia in the brain, alveolar macrophages in the lungs, and monocytes when immature in the circulation). The extreme diversity of macrophage functioning is beyond the scope of the present discussion; however, macrophages are essentially agranular, adherent cells containing one round nuclei and abundant cytoplasm, performing phagocytosis in essentially the same manner as the myeloid cells. In contrast to myeloid cells, they are relatively long-lived and therefore capable of sustained, repeated phagocytic activity. Additional important functions include the processing and

presentation of antigen in preparation for the specific immune responses, amplification and control of immune responses by the release of soluble interleukin mediators, and removal of dead, dying and damaged cells important to the healing process (29).

Another cell present in fish and mammals important to the initial non-specific destruction of invaders are natural killer (NK) cells, which are among the 15% of lymphocytes that do not contain either the B- or T-cell markers. They are large, granular, nonadherent and nonphagocytic lymphocytes, which react through chemotactic factors to destroy foreign cells through killing mechanisms similar to specific T-cell cytotoxicity, and are also very important to the elimination of circulating and solid neoplastic cells (29).

2. Specific Immunity

Fish demonstrate specific, well-defined humoral and cell-mediated immune function, and possess similar major cellular and protein components to that of mammals (5, 13-15, 25). Once a foreign entity is recognized by the first-line defenders, a specific immune response is initiated against the epitopes (cell-surface markers/receptors) of the invader by an interaction with major histocompatibility complexes (MHC I or II), interleukins, and either T-helper cells (Th) or cytotoxic T-cells (Tc) (23-25).

As mentioned previously, the macrophages can also retain a small peptide sequence of the invader to display on their cell surface. Therefore macrophages (and also B-cells, dendritic/langerhans cells, and other IL-1 producing cells) are denoted as antigen presenting cells (APCs), capable of recognizing and presenting antigen to specific immune cells. Following phagytosis of extracellular antigen, small peptide fragments of the invader (10-20 amino acids) are produced, which have high affinity for the MHC II transport protein within the cell. Transport of the antigen-MHC II complex to the surface of the cell allows the Th cell to recognize it as foreign, and a specific antibody-mediated response could be initiated. Th cells cannot recognize antigen unless it is bound by MHC II, which is termed MHC restriction. Alternatively, some APCs are poorly phagocytic, but can bind antigen to cell-surface MHC II, and present to Th cells in this manner (29).

If the antigen is endogenous (derived from inside the cell, such as viral proteins), the protein is fragmented by the cell, but now carries high affinity for MHC I. Transport to the cell surface now allows Tc cells to recognize the foreigner, and a certain cell-mediated response ensues (30).

a. Humoral Immunity

Similar to mammals, humoral immunity in fish targets exogenous foreign material via antibody binding, and is mediated primarily by the B-

lymphocytes--small round cells with a single large, round nucleus and very little cytoplasm. These lymphocytes make up only about 5% of the total lymphocyte population and are relatively short-lived, but are nonetheless very important. Most originate from the bone marrow in mammals, or pronephros in fish, and mature in these organs before migrating to the other primary and secondary lymphoid tissues (5, 13-15, 23-25).

All lymphocytes carry antigenic surface molecules/receptors known as cluster of differentiation (CD) antigens, which denote function and identification. There are over 80 CD antigens described to date in mammals, and most are homologous across mammalian species. As well, most B-cells carry both MHC class I and II antigens, various interleukin receptors, and all B-cells carry a distinct B-cell receptor (BCR -- capable of binding either APC processed or free antigen) and a complement (C') receptor for recognition and response to foreign antigen (24-25).

B-cells initiate body protection after recognition of antigen through the B-cell receptor with appropriate Th help. Remarkably, there is enough variability among the population of lymphocyte specific receptors that nearly any type of foreign invader can be recognized by the immune system. When the antigen is presented in conjunction with an MHC II molecule, a CD4+ Th cell can recognize it and bind. Strong binding alone, however, is not sufficient to promote an immune response, and indeed, may lead to tolerance to the antigen. Therefore, IL-1, a very important costimulator molecule released by

APCs is necessary. Under these correct circumstances, the Th cell will then secrete IL-2 and IL-4, which will immediately promote the B-cells to begin a proliferation or clonal selection process, greatly elevating the proportion of B-cells specific for the particular antigen. These cells in turn differentiate into plasma cells (mature B-cells capable of producing large quantities of antibody proteins) or memory cells (B-cells which can very quickly proliferate and produce an even greater amount of antibody upon a subsequent exposure to the same antigen) (27).

Certain antigens are capable of provoking antibody production in the absence of Th cells. These are T-independent antigens and usually are simple repeating polymers, such as *Escherichia coli* lipopolysaccharide (LPS). Because they are repeating polymers, they process multiple identical epitopes and so can cross-link several BCRs at one time. As a result, the effective dose of these epitopes must be relatively large to provide a sufficient stimulus for the proliferation of at least some B cells. Characteristically, T-independent antigens trigger only IgM responses in mammalian B cells and fail to generate memory cells, due to their failure to induce the appropriate interleukins from Th cells, and so cannot trigger the switch from IgM to other isotypes (23).

Antibodies (Ab) are proteins known as immunoglobulins, produced by immature and mature B-cells (plasma cells) in response to each recognized exogenous foreign invader. In mammals there are five main isotypes of immunoglobulin: IgM (mostly stimulated by the primary response), IgG (for

the memory response), IgA (in secretory fluids), IgE (cell-associated, often implicated in allergic reactions), and IgD (on membranes of B-lymphocytes, possibly important in recognition) (5, 24).

In fish, however, only one type of antibody has been described, which is very similar to the mammalian IgM, yet its structure is tetrameric, rather than pentameric (Figure 1.1) (5, 13-16, 26). Interestingly, fish do display an anamnestic (memory) -like response with subsequent antigen exposure, however the response is not as quick and does not confer immunity for as long. Memory function is normally mediated by IgG antibody in the mammal, but the mechanism for this function in fish is at this point unknown (31-33). It is possible the mediating factors are similar immunoglobulin isotypes that have not been identified, as the kinetics of the fish and mammalian humoral response demonstrated in *in vitro* assays is nearly identical.

When antibodies bind to antigens, forming antigen-antibody (Ag-Ab) complexes, complement-mediated lysis can occur. Complement is composed of a set of protein enzymes whose activation results in a reaction cascade, ultimately leading to the disruption of cell membranes and the destruction of cells or invading microorganisms. There are two pathways of complement activation, the classical and the alternate. The classical pathway requires antibody bound to antigen for activation, while the alternate pathway can be activated against some Gram-negative bacteria containing endotoxin without involving specific antibody (26, 34).

Although both the classic and alternate pathways are present in fish (5, 26, 35), we and other investigators have found that xenogeneic species' complement is not as effective in causing antigen lysis, indicating that there may be major differences between the composition and/or structure of complement components among mammals and fish, and indeed, amongst different fish species (31-33, 35). It is hypothesized that significant structural configuration differences exist to affect binding efficacy between the species.

b. Cell-Mediated Immunity

Cell-mediated immunity (CMI) was a term used originally to describe reactions which were mediated by lymphocytes and phagocytes rather than antibody--localized reactions usually involving intracellular pathogens, cancer or tumor cells, or foreign grafts. However, it is now used in a more general sense for any response against organisms, tumors, or foreign antigen in which antibody plays a subordinate role, including Th-cell dependent and independent reactions, macrophage activation by lymphokines, natural killer cell cytotoxicity, and granuloma formation, as well as cell-mediated cytotoxicity, both antibody independent and dependent (the latter termed ADCC, or antibody dependent cell-mediated cytotoxicity). Antigens and organisms that are inside cells cannot be seen by circulating antibody and complement, therefore these CMI responses are necessary to maintain normal homeostasis of the individual. The detailed discussion of all types of CMI is

beyond the scope of this paper, so our focus will lie in describing the basic mechanism of the T-cell's cytolytic response to foreign antigen, or cell-mediated cytotoxicity (antibody independent).

Cell-mediated cytotoxicity is carried out primarily by T-cells, which are indistinguishable from B-cells morphologically, however they can be defined on the basis of surface receptors or proteins, their ontogeny (where they develop), enzymes produced, and response to molecules (mitogens) that provoke cell division. T-cells mature primarily in the thymus (14, 26, 36) of both fish and mammals.

In mammals a cytolytic response begins when an infected cell uses nonlysosomal proteases to degrade the invader into fragments. The fragments bind with high affinity to MHC I molecules, and the complex is then transported to the surface of the infected cell for presentation. A CD8⁺ T_c cell can now recognize and bind the complex, and in conjunction with Th cells, the T_c cell is prompted to proliferate and mature. Alternately, APC's can present exogenous antigen to Th cells via MHC II as described previously, and the Th cell may stimulate cytotoxic T-cell reactions rather than antibody mediated responses. Thus, the Th cell recognizes particular epitopes and then selects the relevant effector cell function.

The mature T_c cells contain granules containing granzymes and perforins, which immediately and quickly deliver a single "lethal hit" to the infected cells, causing organelles and nuclei to disrupt simultaneously. In the case of

neoplastic cells, the Tc cells secrete tumor necrosis factor- β (TNF- β), which initiates mitochondrial and nuclear fragmentation and cell death ensues. This lethal sequence of events involves three steps: cytoplasmic reorganization of Tc organelles toward the target, target membrane channel formation, and increase of target cell intracellular calcium (36).

To date, very little has been found to compare the cytolytic T-cell immune response between mammals and fish, however, the synthesis of fish monoclonal antibodies to differentiate cell types and function is in its infancy (26, 37). It is known that the thymus acts as a secondary hematopoietic organ in fish as in mammals and is the major source of T-lineage cells (38). As well, several types of interleukins have been found to be similar in structure and function to the mammalian counterparts (26), and all fish species studied respond to concanvalin A (Con A) by growth and differentiation of T-cells that perform in the same manner as mammalian T-cells (39-40). Further, fish produce a strong rejection to allografts and respond in mixed-lymphocyte reaction (MLR) assays in much the same manner as mammals, which is strongly suggestive of the presence of MHC restricted T-cell responses in fish (41-46).

B. The National Toxicology Program's Development of Immunotoxicity Risk Assessment Standards

In the late 1970's and early 80's, there was a rise in scientific interest focusing on the relationship between various pollutants in our environment and their coincidence with increased disease processes. Repeated studies documented the increased incidence of immunosuppression and cancer within animal populations exposed to sub-lethal doses of contaminants (47-48). Consequently, laboratories around the world were developing assay protocols for immunotoxicity testing; however, there was a myrid of variation from laboratory to laboratory, and often the results did not include specific indicators of actual host resistance to pathogens (3).

In 1988, Luster et al. at the National Toxicology Program laboratories used a ten-year compilation of data to develop a specific, tier-wise battery of tests for detecting potential immunotoxic compounds in a well defined rodent model, which was both reproducible and highly predictive of actual immunosuppression (3).

Tier I of the series was designed to identify potential immunotoxicants at dose levels which did not produce overt pathological effects, and consisted of leukocyte and total cellularity counts, tissue to body weight ratios, lymphoid organ histology, antibody production via the PFC assay, lymphocyte blastogenesis via mitogens, the mixed leukocyte response (MLR), and natural

killer cell activity. Tier II further defined the immunotoxic effect by using more specific humoral and cell-mediated assays, and distinguishing the cell type(s) affected. These tests included quantitation of splenic B and T lymphocytes using surface markers, the IgG antibody response using the PFC, assessment of cytotoxicity via either the CTL or delayed hypersensitivity response (DHR), and actual host resistance using syngeneic tumor cells, bacteria, viruses, or parasites (Figure 1.2)(4).

By 1992, the standard assays in Luster's model were used in a cohesive effort between the National Toxicology Program (NTP), the National Institute of Environmental Health Sciences (NIEHS), and the Chemical Industry Institute of Toxicology to generate a database of immunotoxicity analysis for 50 select compounds, in an effort to determine which tests within the tiers had the highest predictive values quantitative immunotoxicity risk assessment (4). The estimated individual and pairwise predictive values of all tests in the tiers have since become the standard by which immunotoxicological studies are based (Figure 1.3).

Briefly, results of these studies indicated that pair-wise use of only 2-3 immune tests with high predictive value for immunosuppression typically provided the same information as gained by using the entire tier (>90% concordance). The most sensitive pairwise indicators of immunosuppression, which gave a 100% predictability with the tested chemicals in the rodent model, were: 1) PFC and CTL; and 2) surface markers and CTL.

The present study incorporates the use the PFC and CTL assays to assess immunosuppression to environmental contaminant, drug, and toxin exposure, as specific monoclonal antibodies to examine immune cell surface marker expression are not as yet available in the tilapian model.

1. The Hemolytic Plaque Forming Assay (PFC)

The direct plaque forming cell (PFC) assay was initially developed by Jerne in 1961, and has since become a mainstay of routine cellular immunology research, primarily accessing humoral IgM antibody responses to antigen (49). Briefly, specific B and T lineage lymphocyte populations previously presented *in vivo* with heterologous erythrocytes are agar plated in combination with the identical erythrocyte suspensions used for immunization. During incubation, the B-cells secrete IgM (in fish, IgM-like) antibody to antigenic determinants present on the surface of the erythrocytes with T-cell help, resulting in antibody-erythrocyte binding. The presence of an adequate complement source allows complement-mediated lysis of the antibody-coated erythrocytes, resulting in the formation of clear zones or “plaques” in the agar (Figure 1.4). Located within the center of each plaque is a single, antibody producing plasma cell. As a lytic site can be produced by a single IgM molecule fixing one C1q complement molecule, high sensitivity is a major advantage of the PFC assay (50).

2. Cytotoxic T-Lymphocyte Assay (CTL)

The cytotoxic T-lymphocyte (CTL) assay assesses the T-cell response to dissimilar MHC antigens, and is the *in vitro* counterpart to the graft versus host (GvH) reaction (51). Basically, lymphocyte cells are purified from the tested animal, and mixed with lymphoblasts, tissue culture cells, or tumor cells which possess dissimilar MHC proteins, i.e., cells from another outbred individual of the same or different species. Previous sensitization of the tested animal with the target cells is not necessary for an adequate response, because as many as 10% of the population of T-cells will respond immediately to the presence of foreign MHC molecules, eliciting a large response (51). Before combining the two cell types, the target cells are exposed to mitomycin C or X-irradiation, which effectively halts DNA replication without causing cell death, therefore only the cytotoxicity (lytic) response of the tested animal is determined (50).

The mixture of cells is then incubated for approximately five days, in order to allow recognition and clonal expansion of the T-cell population. The target cells are then exposed to $^{51}\text{CrO}_2$, which is absorbed into the cytoplasm of the cells without cell damage. Finally, the incubated (stimulated) effector cells and the chromium labeled targets are mixed, and cell lysis of the targets occurs within four hours. The lysis is measured by the amount of gamma radiation that is present in the medium after being released from the lysed cells. To be

certain that the lysis is not due to other *in vitro* factors, maximum and minimum release counts are assessed by incubating either NP-40 or other lysis causing detergents or medium alone with the chromium-labeled targets. The percent of specific release is calculated as follows (51):

$$\% \text{ Specific Release} = 100 \times \frac{\text{CPM}_{\text{exp}} - \text{CPM}_{\text{min}}}{\text{CPM}_{\text{max}} - \text{CPM}_{\text{min}}}$$

C. Environmental Contaminants, Chemicals and Toxins

The following chemicals were specifically selected from the NTP list of rodent immunotoxicants to provide a chemically diverse group inclusive of therapeutic drugs, natural toxins, and examples of the different types of environmental contaminants, such as polycyclic aromatic hydrocarbons, halogenated aromatic hydrocarbons, insecticides, metals, nitrosamines, and solvents.

1. Review of Selected Immunotoxicants for Study

a. Azathioprine (AZA)

Azathioprine is one of the most commonly prescribed immunosuppressive drugs used in the treatment of cancer, and to deter the rejection of transplanted tissue. AZA, or methyl-nitroimidazolyl-6-mercaptopurine (Figure 1.5) is a derivative of 6-mercaptopurine (6MP), but with a

nitroimidazole chain resulting in a longer duration of action than intact 6MP. Since 6MP is phosphorylated intracellularly to thioinosine monophosphate (TMP), and TMP inhibits several enzymes important for purine biosynthesis, rapidly dividing cells are extremely sensitive to the resulting blockade of DNA synthesis (52).

AZA has been proven to affect a variety of immune responses in animals and humans, with the cell-mediated response being more sensitive than the humoral (53). In addition, AZA lowers the total number of lymphocytes in the bone marrow and thymus, and possesses potent anti-inflammatory activity, which is likely to interfere with the expression of various immune responses (53). As well, the risk of developing opportunistic infections, lymphomas, liver tumors, and squamous cell cancers of the skin are high with AZA therapy (54-55).

b. Polycyclic Aromatic Hydrocarbons: Benzo[a]pyrene (B[a]P) and 7-12-dimethylbenz(a)anthracene (DMBA)

Benzo[a]pyrene (B[a]P) and 7-12-dimethylbenz(a)anthracene (DMBA) (Figures 1.6 and 1.7, respectively), of the polycyclic aromatic hydrocarbon (PAH) family of compounds, are documented in the literature as immunotoxic, carcinogenic, and teratogenic in several aquatic species and all mammals examined, including humans (55-63). B[a]P is a nearly ubiquitous environmental contaminant, while DMBA is a synthetic model PAH not

normally found in the environment. Found in coal tar and byproducts of incomplete carbonaceous matter combustion, it is estimated that nearly 900 tons of B[a]P are emitted into the atmosphere yearly in the United States alone. In addition, food may be contaminated by PAH compounds through atmospheric fallout, smoking, grilling, or roasting processes. These toxicants carry an environmental half-life of over 50 years, thus the possibility of immunomodulation in species occupying contaminated regions is of growing concern (64).

PAHs inhibit both cell-mediated and humoral immune responses, however, their exact immunosuppressive mechanism(s) remain largely unknown. Hypotheses include the fact that being structurally planar and highly lipophilic, they are capable of intercalating into plasma or organelle membranes and disrupting transduction of transmembrane signals and/or altering the conformation of membrane receptors. They may also bind the aryl hydrocarbon receptor (AhR) to alter gene expression in a TCDD-like mechanism (see TCDD, page 26). Another proposed mechanism that continues to receive considerable attention is the possibility that PAHs alter the production of various interleukin proteins, specifically IL-1 and IL-2. Further, they have been proven to increase intracellular levels of calcium, leading to DNA fragmentation of B and T cells (64).

c. Cadmium (Cd)

Cadmium is a metal required by biological organisms in ultratrace amounts. Although its primary physiological function is unknown, it has been proven to activate a number of enzymes in a non-specific manner (52).

Metals differ from other toxic substances in that they are neither created nor destroyed, however their utilization influences the potential for health effects via transport through the air, water, soil and food, and by altering the biochemical form of the element. They are among the very first substances discovered to be toxic to man (54).

Cadmium was discovered as an element in 1817. It is a product of zinc and lead mining and smelting, and is a major environmental pollutant. It is used in electroplating or galvanizing due to its noncorrosive properties, as a color pigment for paints and plastics, and as a cathode material for nickel-cadmium batteries (54).

Chronic or acute exposure to Cd affects the phagocytic, humoral and cell-mediated immune responses by depressing hemapoesis in mammals and fish (5, 65-68), although the effects often diminish after elimination of the toxin. Cadmium is also known to cause cancer of the lung and prostate, chronic pulmonary disease, tubular dysfunction of the kidneys, osteoporosis due to elevated calcium excretion, and hypertension (54).

d. Diethylstilbesterol (DES)

Diethylstilbesterol is a synthetic, nonsteroidal estrogen which was widely used in the 1940's to 1970's to prevent miscarriage by stimulating the synthesis of estrogen and progesterone in the placenta (Figure 1.8). Unfortunately, it was determined by the late 1970's that DES exposure in the first trimester of pregnancy led to a high incidence of clear cell adenocarcinoma of the genital tract in young female offspring. In male offspring, a high incidence of epididymal cysts, hypotrophic testes, and capsular induration, low ejaculated semen volume and poor semen quality was observed (54).

DES is a potent cell-mediated immunotoxicant, causing thymic atrophy and a reduction in total thymic cell counts in mammals and fish (5, 64, 69). It is hypothesized that the increased number of estrogen receptors in females account for the more severe incidences of cancer in female animal models, as well as humans. However, the exact mechanism of action is not clear at this time (64).

e. N-nitrosodimethylamine (DMN)

DMN is a member of the N-nitroso family, which are formed as a consequence of the interaction of nitric acid and suitable nitrogen compounds (Figure 1.9). Exposure to DMN comes from a wide variety of dietary and industrial sources, such as cigarette smoke, cheese, seafood, cured meats, alcoholic beverages such as beer and whiskey, and rubber and metalworking

industries (70). Also used as a laboratory solvent, toxicological interest increased in 1954 when cirrhosis of the liver developed in two laboratory workers exposed to DMN (71).

DMN is both toxic and carcinogenic mainly to the liver, but secondary targets include the kidney, lung and esophagus at high levels of exposure. As well, it is a potent immunotoxicant, affecting both humoral and cell-mediated immune responses. As well, investigators recently noted that DMN exposure results in altered production of several inflammatory response cytokines, including IL-1, IL-6, TNF- α , and GM-CSF. The mechanism of action is as yet unknown, however a hypothesis has been put forth that DMN acting locally in the liver results indirectly in the release of soluble inflammatory mediators which affect distant immune target organs (71).

f. Lindane (γ -HCH)

Lindane is one of the most widely used organochlorine insecticides for agricultural purposes in the United States (Figure 1.10), replacing DDT after its restriction in many countries (72). The γ -isomer of hexachlorocyclohexane, lindane is highly lipophilic and thus deposits selectively into adipose tissue (73). Being a very stable compound as well, it is often implicated in chronic aquatic and terrestrial environmental pollution (72-76).

The literature reports inhibition of non-specific, humoral and cell-mediated responses in rodents exposed to lindane (75-76). However,

conflicting studies exist in several fish species, including rainbow trout, carp, and channel catfish, denoting either positive or negative immunotoxicity responses in these species (72-74). In a recent study in tilapia, lindane did not alter phagocytosis or chemiluminescence of phagocytic cells, however, a significant reduction in total splenic and pronephric cell numbers did occur in sub-lethally dosed subjects (72). The mechanism of action of this compound is to date unknown.

g. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

TCDD is a member of the halogenated aromatic hydrocarbon (HAH) family of compounds, including polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs)(Figure 1.11). These highly lipophilic, low-molecular weight molecules are among the most biologically potent toxicants known to man, with TCDD being described as the most toxic chemical ever created (77).

TCDD is not produced commercially (except for laboratory purposes), but is primarily formed as a contaminant during the manufacture of products derived from chlorinated phenols, such as pulp and paper (in the chlorine bleaching process). TCDD is produced as a byproduct in any combustion reaction involving organic materials and chlorine sources, including the combustion of gasoline and industrial wastes and chlorophenoxy herbicide production. Agent Orange, a herbicide used during the Viet Nam war to affect

defoliation, contained trace levels of TCDD as a contaminant. TCDD was also implemented in the most dramatic occurrence of environmental contamination in history, when a safety disk ruptured at a herbicide plant in Sevesco, Italy in 1976, sending a cloud of TCDD-laden herbicide into the air (77).

Well known for its immunotoxic effects in laboratory mammals and fish, TCDD has been reported to cause a number of diverse effects, including a generalized wasting syndrome, pancytopenia, hepatomegaly and hepatotoxicity, chloracne and hyperkeratosis, soft tissue sarcomas, gastric lesions, urinary tract hyperplasia, edema, tumor promotion, teratogenicity and embryotoxicity, decreased spermatogenesis, and psychiatric disturbances (5, 8, 13, 77).

Lymphoid involution, especially of the thymus, is among the earliest and most sensitive manifestations of exposure to TCDD, and is seen universally in all animal species tested. The apparent terminal differentiation of thymic epithelial cells prevents thymocyte maturation, affecting both the cell-mediated as well as the humoral specific immune responses in adult animals. Alterations in normal differentiative processes are seen in most immunocompetent cells. (5, 8, 13, 77-80).

The most studied biological endpoint for TCDD toxicity is the induction of the liver enzyme arylhydrocarbon hydroxylase (AHH), which has led to the elucidation of the primary mechanism of action: TCDD binding of the aryl hydrocarbon receptor (AhR) with subsequent toxic changes to gene products.

In this model, TCDD easily crosses hepatic cell membranes during metabolism due to its lipophilicity, binding to an activated cytosolic AhR (AhR bound to one or more heat shock proteins, specifically, Hsp-90). The normal function of the AhR is currently unknown, however, it is known that it is not a steroid hormone receptor as previously speculated, rather, it is a member of a family of basic helix-loop-helix proteins, which include the mammalian AH receptor nuclear translocator (ARNT) proteins. The TCDD-AhR complex binds ARNT with subsequent release of Hsp-90, and is translocated across the nuclear membrane. Close association with the dioxin-responsive enhancer (DRE) region of the DNA results in an increase of transcription genes, which produce several cP450 transcription factors such as CYP1A1, CYP1A2, CYP1B1, some phase II enzymes, and other unidentified mRNAs. These factors subsequently cross the cellular membrane to increase biotransformation of xenobiotics, and alter cell proliferation and differentiation (77, 80-81)(Figure 1.12).

h. T₂ Mycotoxin (T₂-toxin)

Mycotoxins represent a diverse group of over 40 chemicals produced as secondary biological metabolites of over many different types of fungi and molds. Produced by the *Trichoderma* species, T₂-toxin is considered a trichothecene toxin (Figure 1.13), and is among the most potent protein synthesis inhibitors known. Discovered during attempts to isolate antibiotics, T₂-toxin is present in the environment associated with mold spores, but most

often occurs in moldy cereal grains such as corn, barley, rye, wheat, and milo. Since it is highly resistant to agricultural processing, it is often detected in finished human and animal food products (82-84).

It is well documented in veterinary clinical research that trichothecenes, especially T₂-toxin, suppress immune function and induce neoplasms in livestock ingesting levels below those of overt-toxicity. In turn, the predisposition of food-producing animals to infectious disease organisms such as *Salmonella* and *Listeria* thereby increases animal-to-human transmission of trichothecene toxicosis and immunomodulation. Acute exposure causes severe damage to actively dividing cells in bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa. Chronic, low exposure may either decrease or increase the humoral and cell-mediated immune responses, however, overall immunosuppression is marked by increased susceptibility to bacterial or parasitic infection (84). The mechanism of action is assumed to be the capacity of the toxin to bind to ribosomes, causing the inhibition of protein synthesis in immune and other cell types (83-84).

2. Review of Selected Non-Immunotoxicants

a. Acetonitrile (Methyl cyanide)

Acetonitrile (Figure 1.15) is a widely used industrial and laboratory solvent implicated in central nervous system disorders, liver, kidney, respiratory, cardiovascular and gastrointestinal dysfunction. There is no available evidence to indicate that it is immunosuppressive in either mammalian or fish species (54-55).

b. Formaldehyde

Formaldehyde (Figure 1.16) has been widely used as a solvent and fixative agent by laboratories, hospitals, furniture manufacturers, and the textile industry. This substance primarily causes a well-documented irritation of the nasal and conjunctival mucosa. Bronchial constriction and obstructive lung disease has also been noted, and reduced expiratory flow rates in the small airways of formaldehyde-exposed workers is common. In mammals and fish exposed to high levels of formaldehyde, immunosuppression has not been observed (5, 13, 85-87).

c. Oxymethalone

Oxymethalone is a commonly abused synthetic androgenic-anabolic steroid hormone (Figure 1.16) ingested by athletes to increase physical strength and endurance. In rodent laboratory studies, oxymethalone has been

implicated in causing liver neoplasia and female estrous cycle disruption (54, 88-89). In human psychological studies, it produces subtle mood changes toward aggressive behavior and slowed responses to stimuli (90).

Oxymethalone immunomodulation has not been demonstrated in the mammalian models tested, and literature depicting its testing in fish was not found.

d. Toluene

Toluene, or methylbenzene, is a clear liquid that is primarily produced during petroleum refining, but is also produced in the conversion of coke from coal and as a byproduct in the manufacture of styrene (Figure 1.17). It is used primarily as a raw material in the production of benzene and is backblended into gasoline, as well as being used as a solvent in paints, coating formulations, adhesives, inks, resins, and rubber. There is great potential for exposure of both industrial workers and the general public to toluene because of its use in a wide variety of industrial processes, as well as in a large number of consumer products, including gasoline, dyes, adhesives, cleaning agents, aerosols, paint/varnishes, and swimming pool chemicals (91).

The major health risk associated with toluene is its narcotic effects amongst chemical workers, causing symptoms such as muscular weakness, incoordination, and mental confusion. At high doses, it causes narcosis within

thirty minutes, and may be lethal with longer exposures. There is no evidence of carcinogenicity (91).

Although the NTP listed toluene as non-immunosuppressive in their rodent studies (3-4) in 1992, there are more current studies in the literature suggesting that toluene may exert immunomodulating mechanisms by affecting hemapoiesis by damaging bone marrow cell chromosomes, leading to decreased lymphocyte counts (54). As well, mice exposed to vapor-phase toluene by inhalation exhibited increased susceptibility to respiratory infection with *Streptococcus zooepidemicus* and decreased bactericidal activity against *Klebsiella pneumoniae*, indicating a possible affect on pulmonary macrophage activity (92). Further, mice recently exposed to toluene in their drinking water displayed suppressed lymphoproliferative responses to Con A, PHA, pokeweed mitogen and LPS, depressed PFC, MLR and IL-2 responses, and thymic involution (91).

e. Tert-butylhydroquinone (TBHQ)

Hydroquinones are components found in many household and laboratory products, including paints/varnishes, photographic developers, aerosols, deodorizers, dermatological preparations, motor fuels and oil, skin depigmentation agents, and oxidation and polymerization inhibitors (93). Tert-butylhydroquinone (Figure 1.18) is a metabolite of butylated hydroxyanisole (BHA), a commonly used food preservative with broad biological activities,

including protection against acute toxicity of chemicals and tumor-promoting activities. The literature is sparse regarding the use of tert-butylhydroquinone in research, however there is some evidence to suggest that hydroquinones cause marked increases in tubular cell adenomas of the kidney, hepatocellular neoplasms, mononuclear cell leukemia, tremors, reduced activity, and weight reduction in mice (54). Further, new studies indicate that TBHQ may increase the production of reactive oxygen species in rat and human cells (93-95). No evidence of immunosuppression exists in rodents according to the NTP (3-4), and no literature was found on the use of this chemical in fish immunotoxicity testing.

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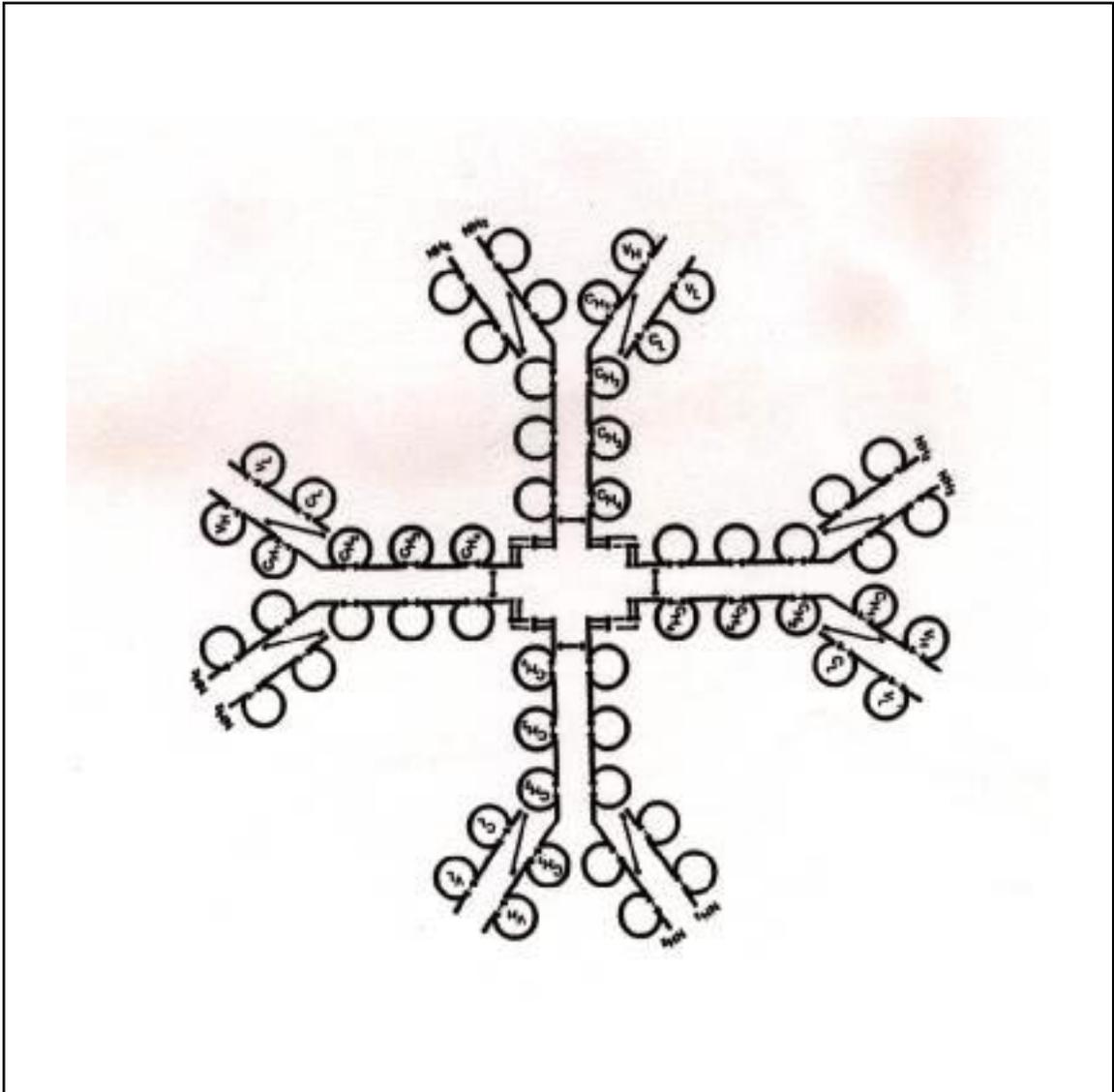


Figure 1.1
Schematic model of the major type of fish immunoglobulin
in its tetrameric form

Adapted from Anderson DP and Zeeman MG (1995). Immunotoxicology in Fish.
In: GM Rand, ed. Fundamentals of Aquatic Toxicology: Effects, Environmental Fate,
and Risk Assessment. London: Taylor and Francis; Vol. 2, p. 371-404.

Panel for Detecting Immune Alterations following Chemical or Drug Exposure in Rodents*		
Procedures	Reference for original method	Reference for NTP modification [†]
TIER I		
Hematology (e.g., leukocyte counts)		
Weights—Body, spleen, thymus, kidney, liver		
Cellularity—Spleen, bone marrow		
Histology of lymphoid organ		
IgM antibody plaque-forming cells (PFCs)	Cunningham <i>et al.</i> , 1965	Dean <i>et al.</i> , 1989
Lymphocyte blastogenesis	Anderson <i>et al.</i> , 1972	Dean <i>et al.</i> , 1989
T cell mitogens (PHA, Con A)	Bach and Voynow, 1966	
T cell (mixed leukocyte response MLR)		
B cell (lipopolysaccharide, LPS)		
Natural killer (NK) cell activity	Brunner <i>et al.</i> , 1976	
TIER II		
Quantitation of splenic B and T lymphocytes (surface markers)		
Enumeration of IgG antibody PFC response [‡]		
Cytotoxic T lymphocyte (CTL) cytotoxicity or delayed hypersensitivity response (DHR)	Lefford, 1974	Holsapple <i>et al.</i> , 1984
Host resistance[§]		
Syngeneic tumor cells		Murray <i>et al.</i> , 1985
PYB6 sarcoma (tumor incidence)		
B16F10 melanoma (lung burden)		
Bacterial models		Bradley <i>et al.</i> , 1985
<i>Listeria monocytogenes</i> (morbidity)		
<i>Streptococcus</i> species (morbidity)		
Viral models		
Influenza (morbidity)		
Parasite models		
<i>Plasmodium juxifii</i> (parasitemia)		

* The testing panel was developed using B6C3F1 mice.
[†] Given if modified from original reference.
[‡] Only a limited number of tests were conducted for this response and are not included in the present analysis.
[§] For any particular chemical tested only one or two host resistance models were selected for examination.

Figure 1.2
 The National Toxicology Program's panel for detecting immune alterations following chemical or drug exposure in rodents

Luster MI, Portier C, Pair DG, White Jr. KL, Gennings C, Munson AE, and Rosenthal, GJ (1992). Risk assessment in immunotoxicology. I: Sensitivity and predictability of immune tests. *Fundamental and Applied Toxicology*. 18:200-210.

Plaque Forming Cells	78 (45)	P=0.0001											
NK Cell Activity	94 (34)	69 (36)	P=0.0014										
T Cell Mitogens	85 (40)	79 (34)	67 (46)	P=0.0003									
MLR	82 (34)	74 (31)	73 (37)	58 (39)	P=0.0458								
DHR	89 (27)	84 (19)	82 (28)	74 (23)	57 (30)	P=0.0348							
CTL	100 (8)	78 (9)	71 (7)	75 (8)	- (0)	67 (9)	P=0.2380						
Surface Markers	91 (23)	90 (21)	92 (24)	87 (23)	93 (14)	100 (5)	83 (24)	P=0.0017					
Leukocyte Counts	86 (28)	71 (24)	62 (29)	59 (27)	67 (18)	67 (8)	80 (20)	43 (30)	P=0.4480				
Thymus/BW Ratio	92 (38)	81 (31)	83 (36)	77 (30)	75 (24)	71 (7)	90 (21)	72 (29)	68 (40)	P=0.0008			
Spleen/BW Ratio	85 (39)	75 (32)	76 (37)	65 (31)	71 (24)	75 (8)	86 (22)	62 (29)	73 (40)	61 (41)	P=0.0395		
Spleen Cellularity	80 (35)	72 (29)	72 (32)	63 (30)	67 (21)	71 (7)	78 (21)	60 (25)	75 (32)	63 (32)	58 (36)	P=0.0684	
LPS Response	81 (37)	73 (30)	69 (39)	65 (31)	58 (24)	63 (8)	90 (20)	56 (27)	74 (34)	71 (35)	63 (27)	50 (40)	P=0.2260
	Plaque Forming Cells	NK Cell Activity	T Cell Mitogens	MLR	DHR	CTL	Surface Markers	Leukocyte Counts	Thymus/BW Ratio	Spleen/BW Ratio	Spleen Cellularity	LPS Response	

Figure 1.3
The National Toxicology Program's immune panel
assessing individual and pairwise concordance
establishing predictability of immunosuppression

Luster MI, Portier C, Pair DG, White Jr. KL, Gennings C, Munson AE, and Rosenthal, GJ (1992).
Risk assessment in immunotoxicology. I: Sensitivity and predictability of immune tests.
Fundamental and Applied Toxicology. 18:200-210.

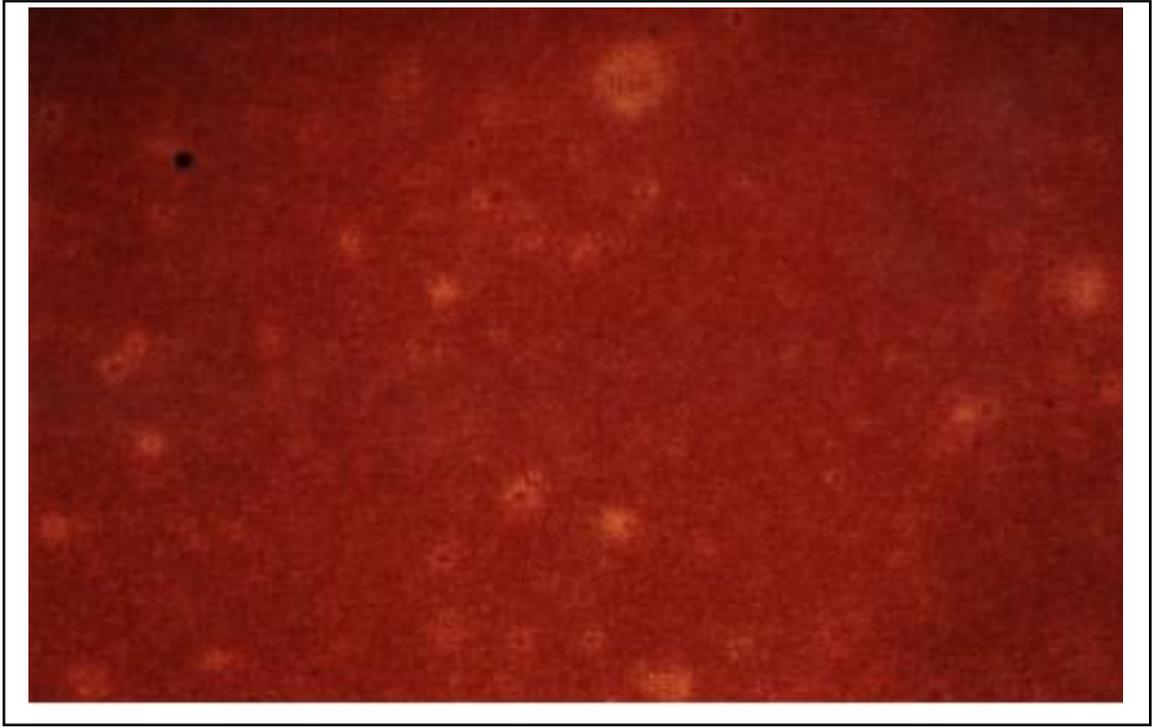


Figure 1.4
Plaque formation in SRBC agar

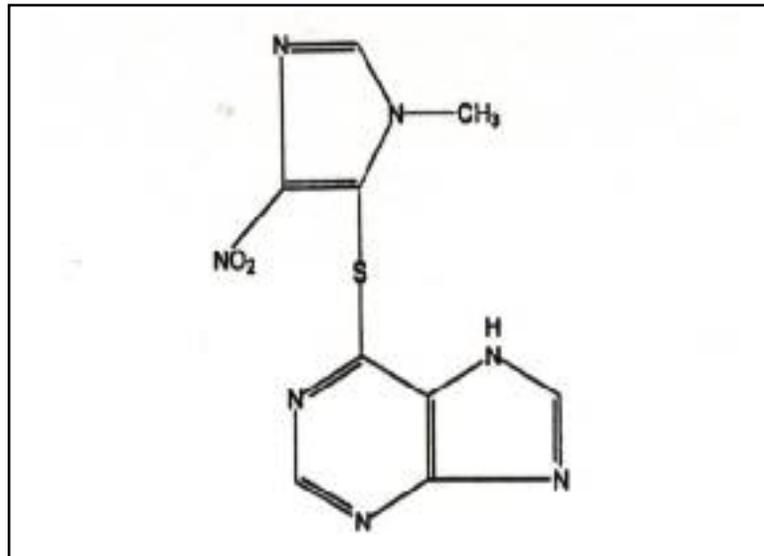


Figure 1.5
Structure of Azathioprine (Aza)

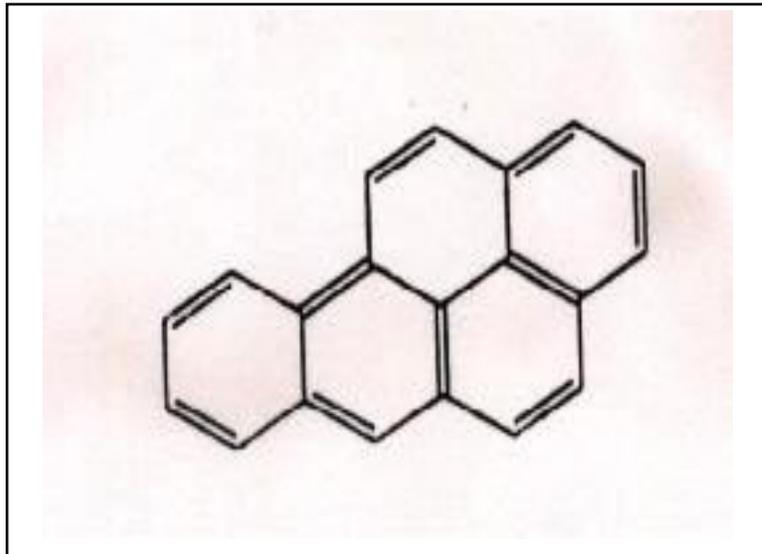


Figure 1.6
Structure of Benzo[a]Pyrene (B[a]P)

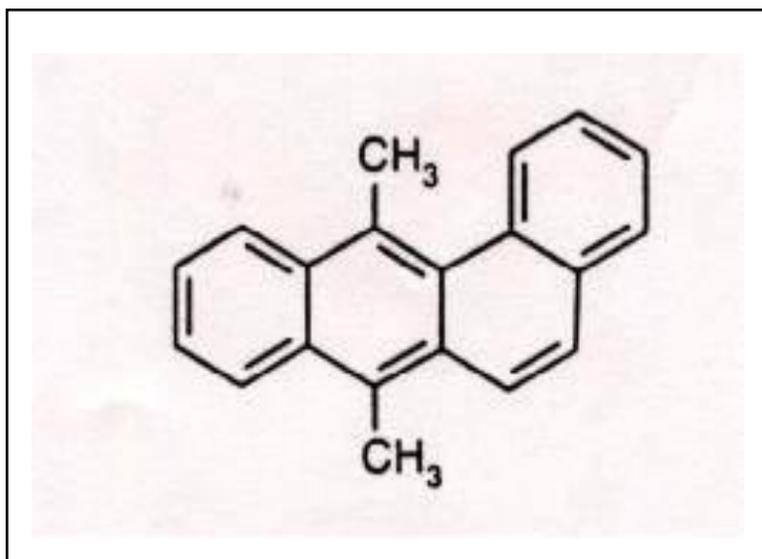


Figure 1.7
Structure of 7-12-dimethylbenz(a)anthracene (DMBA)

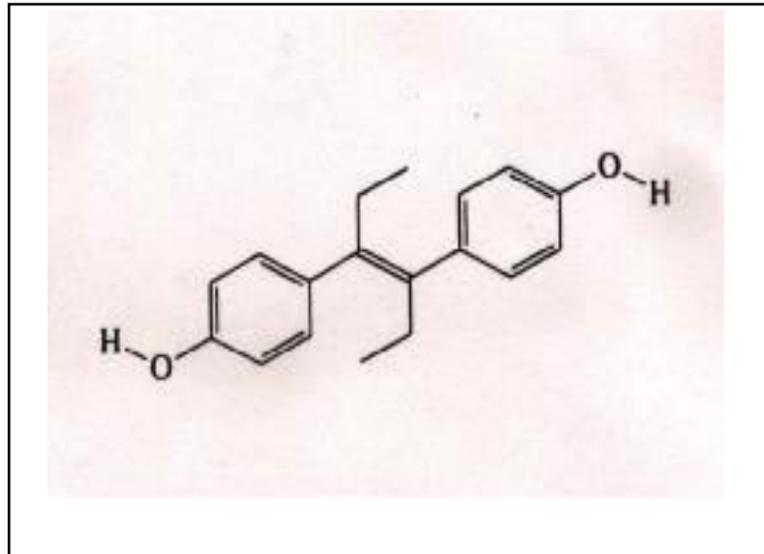


Figure 1.8
Structure of Diethylstilbestrol (DES)

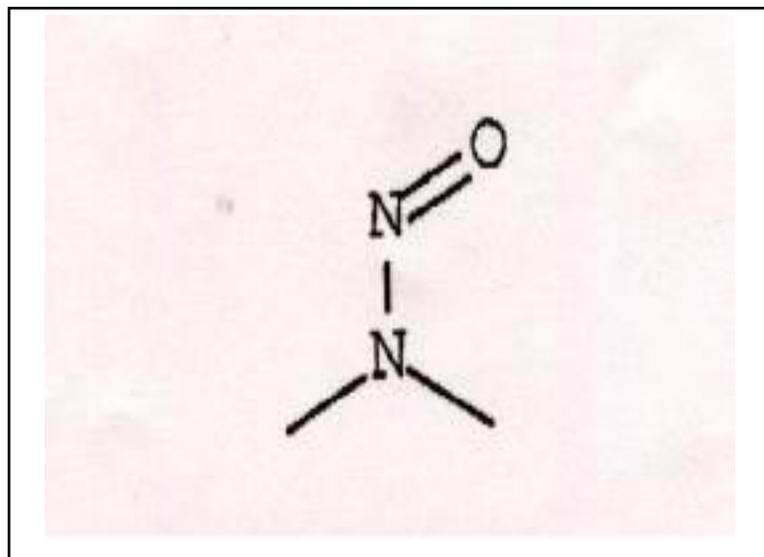


Figure 1.9
Structure of N-nitrosodimethylamine (DMN)

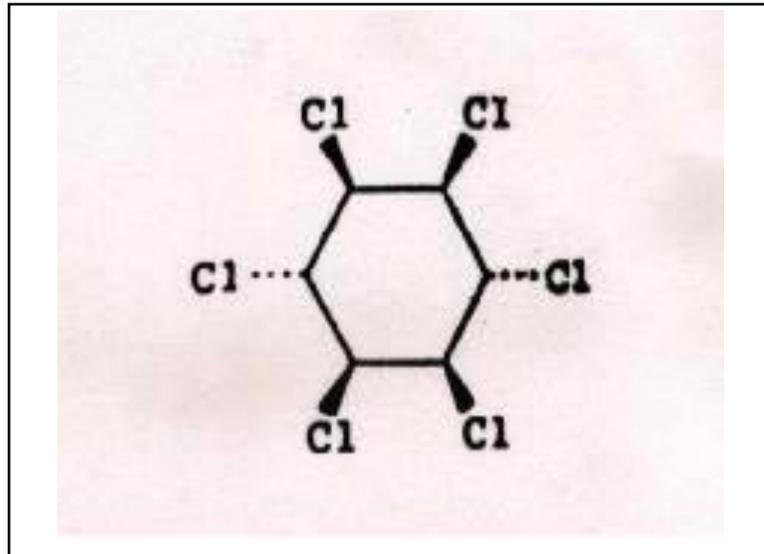


Figure 1.10
Structure of Lindane (γ -HCH)

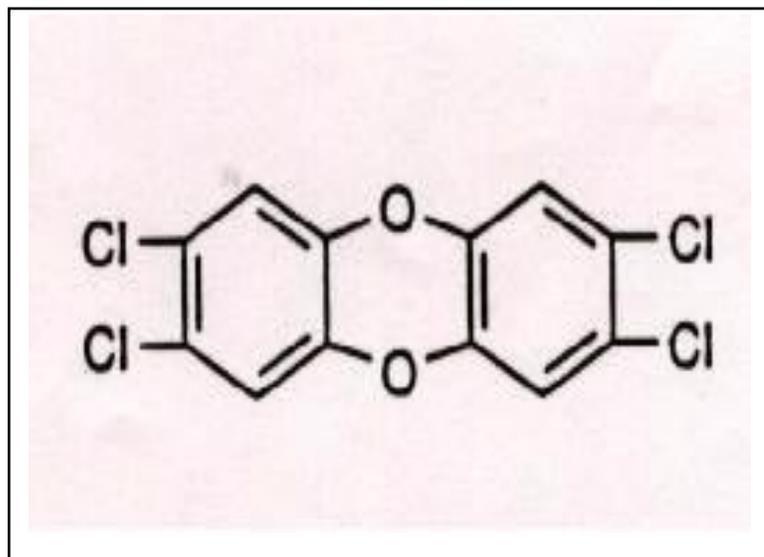


Figure 1.11
Structure of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

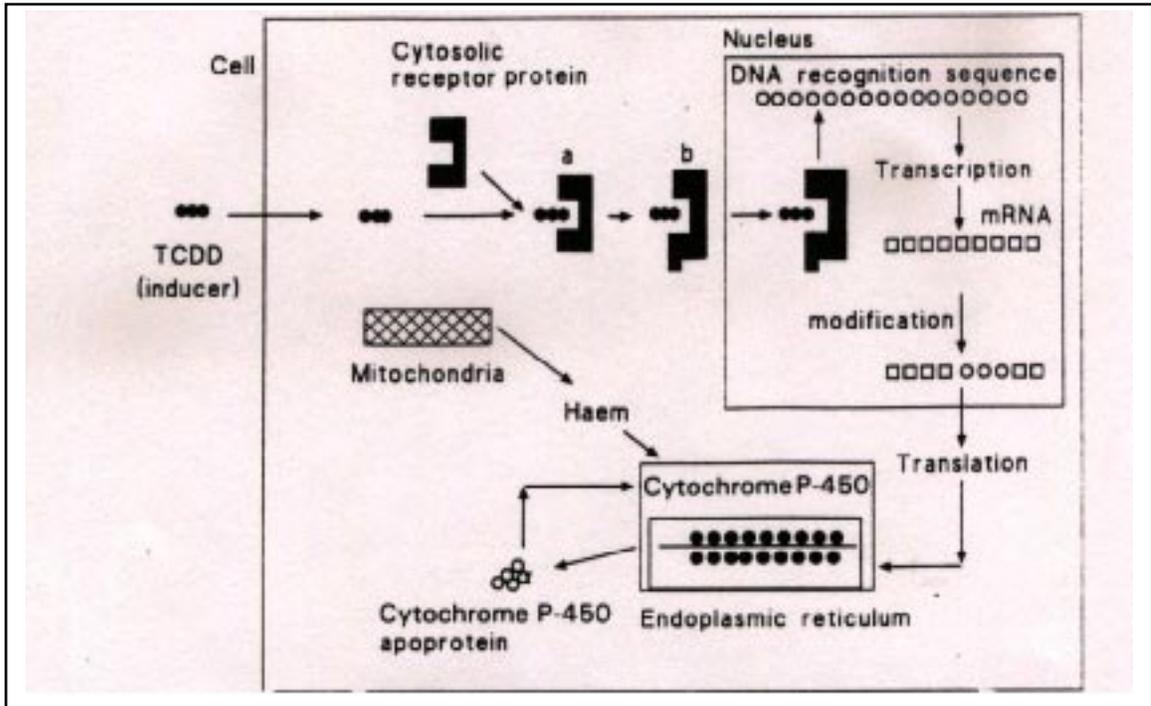


Figure 1.12
Proposed Toxic Mechanism of TCDD

Timbrel JA (1991). Principles of Biochemical Toxicology. Taylor & Francis, Washington D.C.; p. 179.

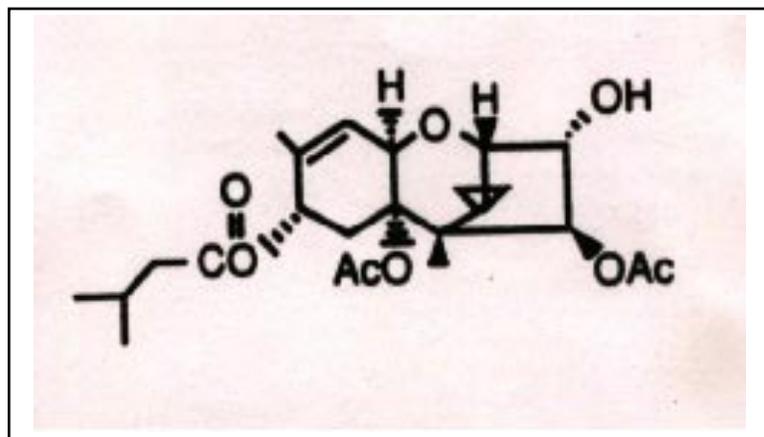


Figure 1.13
Structure of T₂ Mycotoxin (T₂)

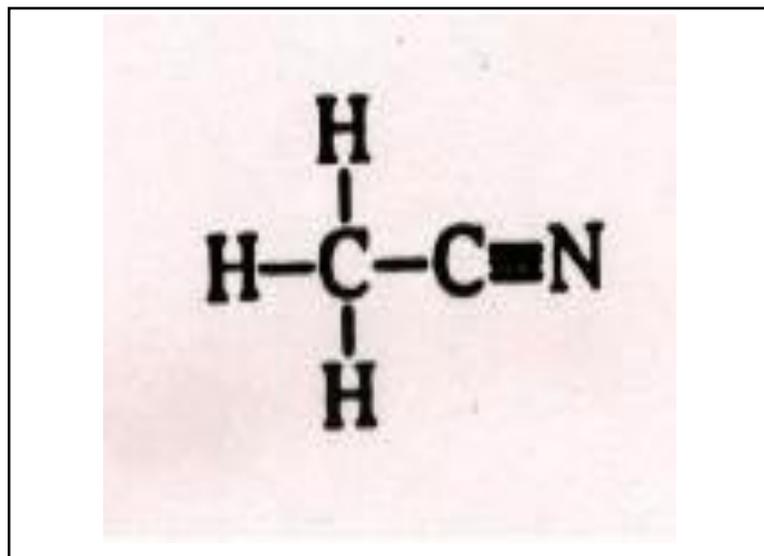


Figure 1.14
Structure of Acetonitrile

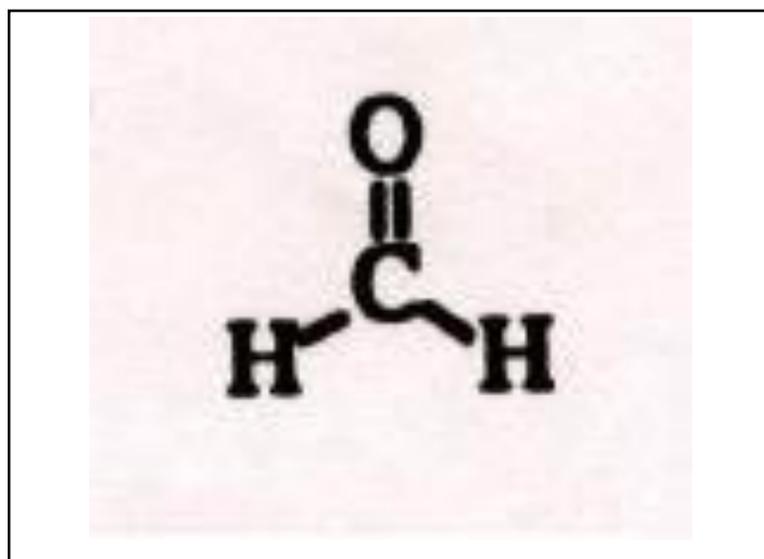


Figure 1.15
Structure of Formaldehyde

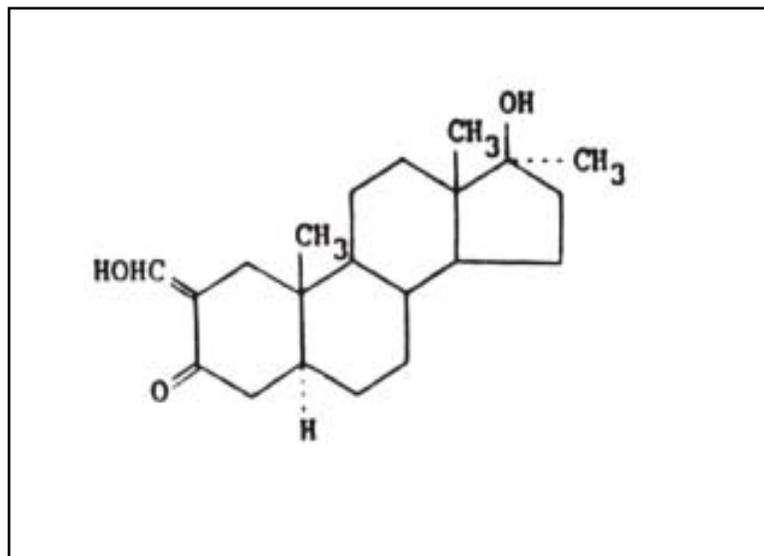


Figure 1.16
Structure of Oxymethalone

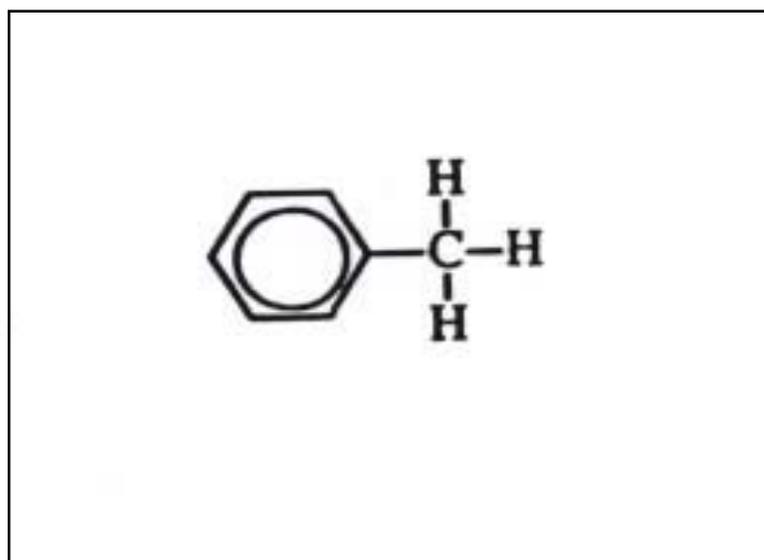


Figure 1.17
Structure of Toluene

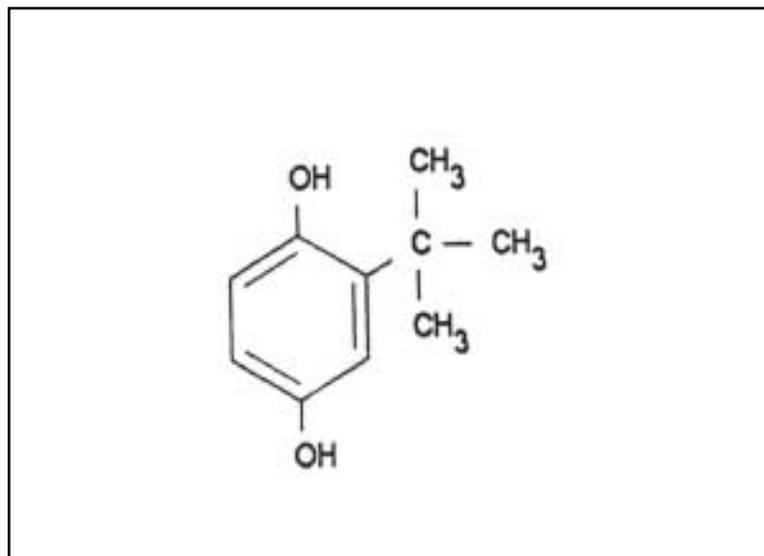


Figure 1.18
Structure of Tert-butylhydroquinone (TBHQ)

MANUSCRIPT I*: The Hemolytic Plaque Forming Cell Assay in Tilapia (*Oreochromis niloticus*) exposed to Benzo[a]Pyrene: Enhanced or Depressed Plaque Formation Depends on Dosing Schedule

Smith DA, Schurig GG, Smith SA, and Holladay SD

(*Submitted to *Journal of Aquatic Animal Health*)

ABSTRACT

The prospect of utilizing the cichlid teleost tilapia (*Oreochromis niloticus*) as an alternative experimental model to mammals for immunotoxicity risk assessment is currently being proposed. As such, the National Toxicology Program's standard battery of rodent immunotoxicity assays is being developed for use in tilapia. Included in this testing series is the hemolytic plaque forming cell assay (PFC), a quantitative indicator of antibody production and exposure to immunotoxic chemicals. Reduced antibody production against sheep red blood cell (SRBC) antigen in response to exposure of fish to the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (B[a]P) was observed using the PFC assay, via reduction in plaque numbers. Under specific immunization circumstances, however, immunostimulation (also a response to immunotoxicity) in chemical-exposed fish was noted via an increase in plaque numbers. Although the normal teleost immune responsiveness was weaker than seen with mice under comparable conditions (presumably due to differences in antibody structure of teleosts), tilapia were

found to exhibit a well-defined primary and secondary humoral response to SRBC, and an immunotoxic response to B[a]P comparable to the rodent model.

Key Words: tilapia; humoral immunity; benzo[a]pyrene; risk assessment

INTRODUCTION

Recent documents prepared by the National Research Council (1992) and the Office of Technology Assessment (1991) focusing on immunotoxicity have cited growing concern within the scientific and public communities regarding the prevalence of toxic agents in the environment and the subsequent increase of immune function impairment and disease. Additionally, the political climate of the last decade concerning the use of mammalian models in toxicity research has promoted the development of assays in alternative, non-mammalian species. As such, we have endeavored to comprehensively examine the development of the National Toxicology Program's standard battery of immunotoxicity assays (Luster et al., 1988; 1992) in the teleost tilapia, and compare the predictive values of immunosuppression obtained to the standard B6C3F1 mouse model.

The direct plaque forming assay was initially developed by Jerne in 1961, and has since become a mainstay of routine cellular immunology research, primarily accessing humoral IgM antibody responses to antigen (Roitt

et al., 1992). Briefly, B and T lineage lymphocyte populations previously presented *in vivo* with heterologous erythrocytes are agar plated in combination with the identical erythrocyte suspensions used for immunization. During incubation, the B cells secrete IgM antibody to antigenic determinants present on the surface of the erythrocytes often with T-cell help, resulting in antibody-erythrocyte binding. The presence of an adequate complement source allows complement-mediated lysis of the antibody-coated erythrocytes, resulting in the formation of clear zones or "plaques" in the agar. Located within the center of each plaque is a single, antibody producing plasma cell. As a lytic site can be produced by a single IgM molecule fixing one C1q complement molecule, high sensitivity is a major advantage of the PFC assay.

The rationales behind the selection of a teleost model for toxic risk assessment include the fact that fish provide an exposure model relevant to natural settings where they are bathed continuously in polluted aquatic environments (Anderson and Zeeman, 1995). Moreover, it has been confirmed by numerous studies that fish have immune processes and metabolic capacities comparable to mammals, possessing similar critical biochemical pathways involved in xenobiotic chemical conjugation, detoxification and activation, and DNA adduct formation and repair (Potter et al., 1994; Stegeman and Hahn, 1994; Ostrander, 1996; Leubke et al., 1997). As an example, cytochrome P450 liver microsome induction by PAH has been demonstrated in tilapia hybrids and other fish species (Ueng et al., 1994; Ueng and Ueng, 1995; van Veld et al., 1997).

These studies and others have promoted the scientific acceptance of fish as viable research models.

Tilapia were identified as a potential fish model for the present series of chemical immunotoxicity studies for several regions. First, the aquaculture of tilapia as a food source is ranked second and fourth for the world and the United States, respectively. As it is common aquaculture practice to use water fed by possibly contaminated natural sources, assessment of the specific immunotoxic responses in tilapia has worldwide health and economic importance. Second, at an average adult weight of 100-250+g, tilapia offer the advantage of larger tissue mass and greater lymphocyte numbers as compared to the other fish models used in toxicity testing, such as the fathead minnow (*Pimephales promelas*) and medaka (*Oryzias latipes*). Third, tilapia are easily bred and maintained in the laboratory setting. Finally, tilapia are inexpensive, currently costing approximately \$0.75 each compared to an average cost of about \$13 each for B6C3F1 mice.

For the present studies, the rodent PFC assay was first modified for use in tilapia. Subsequently, the PFC assay was evaluated in tilapia exposed to the PAH B[a]P. Benzo[a]pyrene is a nearly ubiquitous environmental contaminant documented in the literature as an immunotoxicant and carcinogen in several aquatic species and all mammals examined, including humans (Thornton et al., 1982; Melius, 1984; Hohn-Bentz et al., 1983; Stegeman et al., 1984; Romero et al., 1991; Yaun et al., 1994; Krieger et al., 1995; Holsapple et al., 1995; Davila et al.,

1996). Found in coal tar and a byproduct of incomplete carbonaceous matter combustion, it is estimated that nearly 900 tons of B[a]P are emitted into the atmosphere yearly in the United States alone (Murray and Thomas, 1992).

Considering the toxicant carries an environmental half-life of over 50 years, the possibility of immunomodulation in exposed species is of growing concern, and thus, the need for new immunotoxicity testing methodologies is increasing.

MATERIALS AND METHODS

ANIMALS. Tilapia were produced in-house at the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) and at the Aquaculture Center of Virginia Polytechnic Institute and State University. Fish of approximately the same age and average weights of 165.3 ± 5.7 g were arbitrarily selected, then transferred individually to three separate compartments in 80 liter long tanks with filtered, aerated, and dechlorinated water. Temperature and lighting conditions were maintained at 26 °C with a 12/12 light/dark cycle. Ammonia and nitrate levels were monitored weekly, and tanks were cleaned as needed. Fish were fed a commercial fish diet of floating nuggets or brood stock pellets (Ziegler Bros., Inc., Gardners, PA) at 2% body weight every other day, and were allowed a two-week acclimation period before each experiment.

REAGENTS. Hank's buffered saline solution (HBSS) was prepared fresh (Hudson and Hay, 1989) and adjusted to pH 7.3 with either 1N HCl or 1N NaOH, then filter sterilized. Complete media was prepared fresh with RPMI 1640 containing hepes (25 mmol) and L-glutamine (2 mmol) (Mediatech, Fisher Scientific, Fair Lawn, NJ) with the addition of 50 IU/ml penicillin (ICN, Costa Mesa, CA), 50 mg/ml streptomycin (ICN) and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Trypan blue (Flow Laboratories, Fisher Scientific) used for differentiation, enumeration, and viability of lymphocytes was diluted 1:10 in phosphate buffered saline (PBS: Sigma Chemical Company, St. Louis, MO). Modified Wright Stain (Sigma) was used for differentiation of cells on cytopsin slides. Bacteriological agar (Sigma) was layered in 5 cm petri dishes (Fisher) to create a level surface for antibody production at 1.4%. Bacteriological agar at 0.7% was used for suspension of lymphocytes and SRBC (Hudson and Hay, 1989). Diethylaminoethyl-dextran (DEAE, Sigma) was used to avoid DNA perturbation in the 0.7% agar suspension. A 15% solution of fetal bovine serum (Sigma) was used to facilitate adherence of cells to slides during the cytopsin technique. Permout® (Fisher) was used to protect cells on the cytopsin slides. Tricaine methanesulfonate (MS-222; Sigma) was used in instances where fish anesthesia was necessary at a concentration of 1.2 g per 6.0 liter of water.

HEMAGGLUTINATION ASSAYS. Prior to PFC dosing, the dilution and dosage of SRBC antigen required to produce an adequate *in vivo* humoral response detectable in the peripheral blood of tilapia was determined by injecting the fish with 2%, 10%, 20% and 50% SRBC at 0.1, 0.2, and 0.5 ml per 100 g of body weight, and performing successive hemagglutination assays to determine the kinetics of the peripheral blood antibody titer.

Fish were bled via caudal venal puncture with one-inch 21-gauge needles and tuberculin syringes (0.5 ml obtained). Blood was transferred to 3 ml red-topped Vacutainers® (Fisher) without additives and allowed to clot at room temperature for 45 min. The clots were not allowed to adhere to the slides of the tubes. The tubes were then transferred to ice for one hr to facilitate complete serum separation, and centrifuged at 400 × g, 7 min, 4 °C. Serum was collected via Pasteur pipette, then heat-inactivated components for 30 min at 56 °C to destroy complement. In a 96 well round bottom cell culture plate, 50 ml PBS were added to all experimental wells. Fifty microliters of each serum sample were added to separate wells containing the PBS, giving a 1:2 dilution. Additionally, dilutions of each sample were repeated to a 1:65,536 serum:PBS dilution. Fifty microliters of 0.5% SRBC were then added to all wells. The plate was covered and sealed with Parafilm® wrap (Fisher) to keep out moisture, and incubated in a water bath at 30 °C for one hr.

Peripheral blood antibody titers were also assessed via hemagglutination on the day of the PFC assay to determine if specific

circulating antibody was present at that time. Prior to sacrificing, fish were bled to a collected volume of 0.5 ml, and the procedure used for the initial hemagglutination assay described above was followed.

CHEMICALS AND DOSING. Dosing solutions were prepared by dissolving B[a]P (Sigma) in corn oil with gentle heat at approximately 60 °C with periodic stirring until completely dissolved, then stored in the dark at room temperature. Fish were dosed with a volume of 0.10 to 0.20 ml. Sheep blood was obtained from one of two sheep housed at VMRCVM in 500 ml sterile bottles with sodium citrate added as an anticoagulant. Fifteen milliliters of sheep blood were washed in HBSS three times at 400 × g, 10 min, 4 °C, and the resulting pellet of SRBC was diluted to 20% in HBSS and stored at 4 °C.

All chemical dosings were delivered via intraperitoneal injection using tuberculin syringes and 25-gauge needles. Experiments were designed at the onset as randomized complete blocks following the procedure of Sokal and Rohlf (1995). Briefly, five sets of three fish each were sampled (15 total, N=5 per dosing group). Within each set of three fish, two were injected with 0.2 ml/100 g body weight of 20% SRBC on day 1 and day 14 to assess the primary and subsequent secondary response to the SRBC immunogen, and one was injected with 0.2 ml/100 g body weight of HBSS only for a negative control. B[a]P was injected into one fish of the SRBC dosed group on days 14, 16, and 18, and vehicle only (corn oil) was given to the remaining SRBC dosed fish.

Summarizing, for each experiment, one fish received SRBC plus B[a]P, one fish received SRBC plus vehicle only for a positive comparative control, and one fish received HBSS only without SRBC for a negative control. Lymphocytes were collected from the immunopoietic pronephros (anterior kidney) on day 21 (seven days post-secondary immunization) (Table 1).

In a second set of experiments fish were again injected as above with 0.2 ml/100 g body weight of SRBC on days 1 and 14. In these experiments, however, B[a]P was injected as a single dose between the primary and secondary immunizations of fish with SRBC. Specifically, on day 7, three fish received an injection of B[a]P, and three fish received vehicle only (corn oil). Summarizing, three fish received SRBC plus B[a]P and three fish received SRBC plus vehicle only as positive controls. Negative PFC responses during the initial experiments deemed negative control fish (those without SRBC or B[a]P) as being no longer necessary. Lymphocytes were again collected from the pronephros on day 21 (seven days post-secondary immunization) (Table 2).

CELL PREPARATION AND ENUMERATION. Anesthetized fish were sacrificed via cranial blow, weighed, and placed on ice. The pronephros tissue was excised under sterile conditions and immediately placed into 5 ml of cold HBSS in sterile plastic 7 cm petri dishes (Fisher). Pronephric cells were separated by gentle maceration over 60 mm autoclavable wire mesh screening, transferred to 15 ml conical tubes (Fisher), and washed one time in cold HBSS at

400 x g, 7 min, 4 °C. The resulting cellular pellet was resuspended in 4 ml cold complete medium and placed on ice. A 50 ml aliquot was removed and combined with 450 ml 10% trypan blue for lymphocyte differentiation, enumeration and viability assessment in a hemacytometer at 40X. Each fish sample was diluted to 40.0×10^6 lymphocytes per ml in complete medium, and stored on ice.

COMPLEMENT PREPARATION. Homologous tilapia blood serum was used as a compatible complement source. For each experiment, two large tilapia (approximately 500 g) were anesthetized using MS-222 and aseptically bled via caudal vena puncture, obtaining a total volume of 3 ml per fish. The blood was immediately transferred to two 3 ml red-topped glass Vacutainers, without additives and the serum was separated in the same manner as for the hemagglutination assays previously described. Serum was collected via Pasteur pipette, diluted to 10% in cold HBSS, and stored on ice for no longer than 30 min before use to avoid denaturation of the complement components.

AGAR AND PFC ASSAY PREPARATION. Two separate dilutions of agar were necessary. Agar (0.7 g) was combined with 50 ml HBSS at 80 °C with gentle stirring until completely dissolved. Two milliliters of this 1.4% solution were transferred to each petri dish with a warmed 5 ml pipette and allowed to solidify to ensure a level surface in the assay dish. These plates were stored at

4 °C until needed, but for no longer than one week to deter bacterial growth. For lymphocyte and SRBC suspension, a 0.7% solution of agar was prepared by combining 0.35 g of agar plus 0.025 g of DEAE with 50 ml HBSS at 80 °C with gentle stirring until just dissolved, then transferred to a 45 °C water bath. Assay procedures were conducted quickly at this point to avoid agar gel precipitation.

PFC ASSAY AND ENUMERATION. Two-hundred and fifty microliters of each serum sample were combined with 0.15 ml 20% SRBC and 0.80 ml of 45 °C 0.7% agar/DEAE solution, mixed quickly, then poured into the previously prepared petri dishes and allowed to solidify. Plates were incubated for two hrs with humidity at 30 °C to allow specific lymphocyte recognition of SRBC and subsequent antibody production and binding. One milliliter of the 10% complement source serum was then added, and the plates were returned to incubation overnight to allow complement mediated-lysis of the SRBC (plaque formation). Plaques were enumerated and evaluated for approximate size manually via low-powered microscopy using a Nikon AFX-DX dissecting microscope with a 10X ocular strength.

CYTOSPIN TECHNIQUE FOR CELLULAR MORPHOLOGY. After completing the PFC assay, the pronephric lymphocyte samples were diluted to 5×10^6 lymphocytes/ml in HBSS, and a 200 ml aliquot of the cell suspension was added to a cytospin chamber with 50 ml fetal bovine serum and 250 ml of PBS.

The chambers were centrifuged at 200 × g, 7 min, 23 °C. The slides were removed and allowed to air dry for five min before fixing in 95% methanol for two min. Slides were stained in Modified Wright for 40 sec, dipped in distilled water for 40 sec, rinsed thoroughly in distilled water and carefully blotted dry. Slides were covered with two drops of xylene diluted Permount, solution and 25x25 mm glass coverslips (Fisher).

STATISTICAL ANALYSIS. Experiments were designed as randomized complete blocks (a specialized two-way analysis of variance) (Sokal and Rohlf, 1995) and were analyzed using a SAS computer analysis package designed especially for this application, employing cross-wise comparisons using Tukey's analysis, and a significance level of $P < 0.05$. Data presented are representative of three replicate experiments.

RESULTS

All fish which received vehicle and SRBC (positive controls) or HBSS alone (negative controls) presented as clinically normal throughout the dosing regimens. Gross examinations of the pronephros tissue of the positive controls showed numerous darkly pigmented spherical bodies and an overall brownish color as reported by previous SRBC immunization studies in fish (Sailendri and Muthukkaruppan, 1975). The pronephros of the negative control fish appeared more reddish in color and showed no pigmented bodies.

Fish dosed with B[a]P displayed limited signs of stress, including hyperpigmentation of the scales and fins, slight lethargy, and reduced feed intake. Pronephric tissue of all B[a]P dosed fish appeared more friable and dark brownish in color, with numerous pigmented bodies similar to that seen in the positive control fish.

HEMAGGLUTINATION ASSAYS. Tilapia produced a low grade primary humoral response to SRBC, with average titers of 1:64 on days 14 through 21 after the first immunization. However, the strongest peripheral blood antibody response occurred when tilapia were immunized twice intraperitoneally on day 1 and 14 with 20% SRBC at 0.2 ml/100 g, producing a secondary response with an average titer of 1:8,192 from days 12 through 21 after the second immunization. Assays performed post-toxicant exposure on all fish on the day of experimentation (7 days after the second SRBC immunization) showed no agglutination.

DIFFERENTIAL IMMUNE CELL ENUMERATION AND VIABILITY. For the first set of experiments (chemical exposure on days 14, 16, and 18), fish dosed with B[a]P exhibited decreased total pronephric lymphocyte counts as compared to fish dosed with vehicle (**Figure 1**). The second set of chemical exposure conditions (B[a]P on day 7) produced similar depression in total lymphocyte counts as compared to the vehicle dosed fish (**Figure 2**). Viability

of cells was high (>95%) for all treatment groups, with no significant group-related differences. However, leukocytes from all B[a]P dosed fish in both studies displayed atypical morphology, including abnormal cellular shape, broken cellular membranes, and occasional apoptotic bodies, as previously described (Holladay et al., 1998).

PLAQUE ENUMERATION. In fish dosed with B[a]P on experimental days 14, 16, and 18, plaque numbers were increased significantly as compared to the vehicle dosed fish (**Figure 3**). Negative control fish receiving HBSS exhibited no plaque formation on any of the trials. All groups of fish, with the exception of the negative controls, exhibited similar qualitative results of both large and small plaques, with no significant difference in size between the sets.

In fish dosed with B[a]P on experimental day 7, plaque numbers were decreased significantly as compared to vehicle dosed fish (**Figure 4**). As with the first set of experiments, all plaques exhibited similar qualitative results of both large and small plaques.

DISCUSSION

Fish constitute a dissimilar and phylogenetically lower species than rodents, thus the implementation of the hemolytic PFC assay for tilapia necessitated several modifications of parameters employed in the classic mammalian assay. Unlike the mouse, which possesses pentameric IgM and

monomeric IgG antibodies, tilapia possess only a tetrameric immunoglobulin molecule similar to IgM, with no form of IgG described to date. Initially, preliminary hemagglutination assays were required to determine the kinetics of the tilapian peripheral blood Ig response, in order to determine the optimal SRBC immunization schedule. These tests showed peak antibody titers to SRBC occurring between days 12-21 post-secondary immunization in peripheral blood.

The spleen is the tissue of choice for providing lymphocytes in murine PFC assays, however, in tilapia this organ proved to be a relatively poor source of these effector cells. Specifically, the tilapia spleen was found to contain only approximately 3.1×10^3 lymphocytes/mg, compared to approximately 3.3×10^5 lymphocytes/mg in a young adult C57Bl/6N mouse spleen (unpublished data). Two other potential sources of lymphocytes in the fish, the peripheral blood and the pronephros, were also evaluated during the course of PFC assay development. Of all three, the pronephros consistently provided the greatest number of lymphocytes. Previous reports in other fish species, where lymphoproliferative responses were examined as indicators of environmental chemical exposure, also found the pronephros to be the optimal source of lymphocytes, supporting the present observation in tilapia (Sailendri and Muthukkaruppan, 1975; Anderson et al., 1979, 1990; Spitsburgen et al., 1986). Fish were originally sampled on days 3-18 post SRBC secondary immunization to determine the peak antibody response of pronephros lymphocytes to SRBC.

Responses were found to occur earlier than that of the peripheral blood, specifically from days 5-13 post secondary immunization, with optimal responses occurring on days 5-7.

The decreased antibody affinity and specificity in fish presumably caused by fewer antigen combining sites available on the tetrameric Ig molecule necessitated using increased numbers of lymphocytes for the tilapia PFC assay (40×10^6 cells/ml compared to 20×10^6 cells/ml in the rodent assay).

Mammalian PFC assays are also typically performed using guinea pig or rabbit complement. Such complement produced only minimal plaque formation in tilapia (data not shown), suggesting mammalian complement components at best possess greatly decreased binding affinity with tilapian antibody. In contrast, plaque formation occurred readily when homologous serum was used as a complement source.

Benzo[a]pyrene is a well characterized immunosuppressive PAH, and therefore was chosen to test the new PFC assay in tilapia. This compound has previously been shown to reduce total splenic and pronephric lymphocyte counts in tilapia, and to inhibit phagocytic cell chemiluminescent responses (Hart et al., 1998). In the present report, B[a]P also caused a significant decrease in plaque formation in exposed fish relative to control fish, indicating this carcinogenic PAH suppresses the tilapian humoral immune response. These results in fish are similar to previous reports in rodents where PAH were also found to inhibit PFC responses (Dean et al., 1994).

Interestingly, during assay development it was found that fish exposed to a relatively high dose of B[a]P (25 mg/kg) concurrently with the secondary SRBC immunization on day 14 displayed significantly enhanced PFC responses (i.e., increased plaque numbers). Bice et al. (unpublished data) have similarly seen both increased and decreased PFC responses in rodents dosed with B[a]P, depending on when the B[a]P was administered in relation to the SRBC. As well, Smialowicz et al. (1997) have reported a similar observation in rodents exposed to the halogenated aromatic hydrocarbon PCB153.

Specific mechanisms by which B[a]P or other immunotoxicants may either increase or decrease PFC plaque formation, depending on dosing schedule employed, have not been elucidated. Pallardy et al. (1992) have suggested that PAH may exert immuno-modulatory effect(s) by intercalating into cell membranes and subsequently altering the conformation of receptors within the membrane and/or disrupting transduction of transmembrane signals, an effect which may lead to increased proliferation of immune cells in response to antigen. In this regard, the PAH 7,12-dimethylbenzanthracene has been found to decrease membrane fluidity of resting T lymphocytes (including T cells displaying suppressor activity) in human peripheral blood suspensions (Ladics and White, 1996). Mounho et al. (unpublished data) have further observed that B[a]P and its metabolites stimulate the Lyn and Syk family of kinases associated with antigen receptor signaling in human B cells, thereby increasing the antigenic response to SRBC in the PFC assay. These authors also

postulated that elevated immune cell cyclic AMP levels caused by PAH exposure may contribute to enhancement of humoral immunity in some exposure situations (Burchiel and Melmon, 1979). It may be important to note that, in all the above reports where immunotoxicant agents enhanced humoral immune responses, the immunotoxicant was given concurrently with the SRBC antigen.

In summary, the PFC assay was modified for use in a widely cultured fish species, tilapia. The assay was optimized by characterizing the antibody titer kinetics in peripheral blood and potential lymphocyte-source tissues, and by varying incubation temperature and complement source. The new assay was then tested using a PAH compound known to alter humoral immune function in rodents. This compound (B[a]P) was found to similarly suppress the tilapian PFC response when traditional dosing parameters were used (i.e., when chemical administration was not concurrent with antigen immunization). When chemical treatment was concurrent with antigen exposure, tilapia demonstrated enhanced plaque formation, an effect again seen previously in rodents similarly dosed. These data indicate that tilapia respond in a manner similar to mammals when challenged with known immunotoxic chemicals, and, thus, that fish may have the potential for development as an alternate species to mammals for use in preliminary chemical immunotoxicant detection and immunotoxicity assessment.

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TABLES AND FIGURES

Table 1. Dosing schedule of fish exposed to 25 mg/kg B[a]P on experimental days 14, 16, 18, and SRBC on days 1 and 14 (simultaneous antigen and chemical dosing).

Table 2. Dosing schedule of fish exposed to 15 mg/kg B[a]P on experimental day 7, and SRBC on days 1 and 14 (separate antigen and chemical dosing).

Fig. 1. Pronephros cellularity in tilapia exposed to 25 mg/kg B[a]P on experimental days 14, 16, and 18. Evaluation was on the third day (day 21) following cessation of dosing. Each determination represents the mean \pm SEM. * indicates significantly different from control, $p < 0.05$.

Fig. 2. Pronephros cellularity in tilapia exposed to a single dose of 15 mg/kg B[a]P on experimental day 7. Evaluation was on experimental day 21. Each determination represents the mean \pm SEM. * indicates significantly different from control, $p < 0.05$.

Fig. 3. Effect of B[a]P on the humoral immune response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 25 mg/kg B[a]P on experimental days 14, 16, and 18. Group 1 fish

(negative controls) were immunized with Hank's buffered saline solution (HBSS) only, group 2 fish (vehicle-exposed) with SRBC, and group 3 fish (B[a]P exposed) with SRBC. No plaque formation was observed in fish treated with HBSS only. Enhanced plaque formation was observed in B[a]P treated fish. * indicates significantly different from the Vehicle + SRBC group, $p < 0.05$.

Fig. 4. Effect of B[a]P on the humoral immune response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received a single dose of 15 mg/kg B[a]P on experimental day 7. Vehicle and B[a]P-exposed fish were immunized with SRBC. Inhibited plaque formation was observed in B[a]P treated fish. * indicates significantly different from the Vehicle + SRBC group, $p < 0.05$.

TABLE 1

Dosing schedule of fish exposed to 25 mg/kg B[a]P on experimental days 14, 16, 18, and SRBC on days 1 and 14 (simultaneous antigen and chemical dosing)

Fish Numbers	Day 1	Day 14
1, 4, 7, 10, 13	SRBC	SRBC and B[a]P
2, 5, 8, 11, 14	SRBC	SRBC and Corn Oil
3, 6, 9, 12, 15	HBSS	HBSS and Corn Oil

TABLE 2

Dosing schedule of fish exposed to 15 mg/kg B[a]P on experimental day 7, and SRBC on days 1 and 14 (separate antigen and chemical dosing)

Fish Numbers	Day 1	Day 7	Day 14
16, 18, 21, 23, 25	SRBC	B[a]P	SRBC
17, 19, 22, 24, 26	SRBC	Corn Oil	SRBC

FIGURE 1

Pronephros cellularity in tilapia exposed to 25 mg/kg B[a]P on experimental days 14, 16, and 18

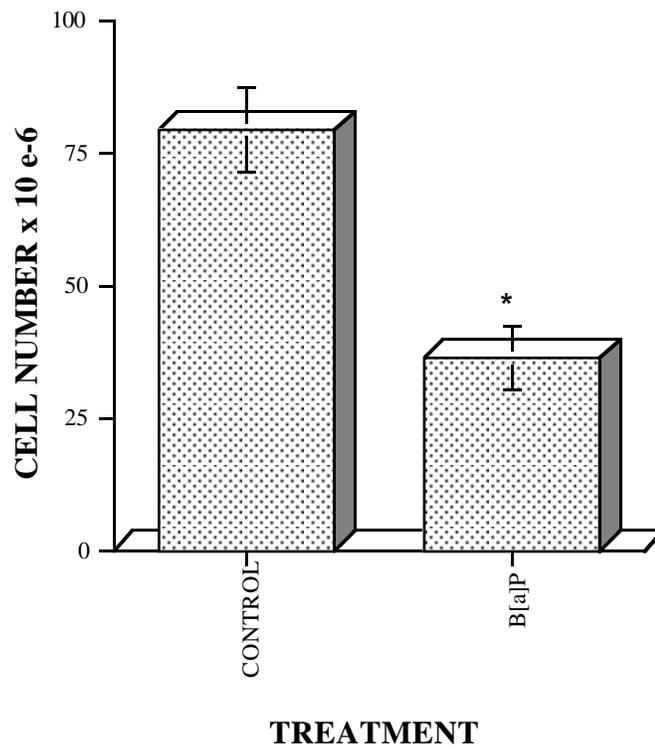


FIGURE 2

Pronephros cellularity in tilapia exposed to a single dose of 15 mg/kg B[a]P on experimental day 7

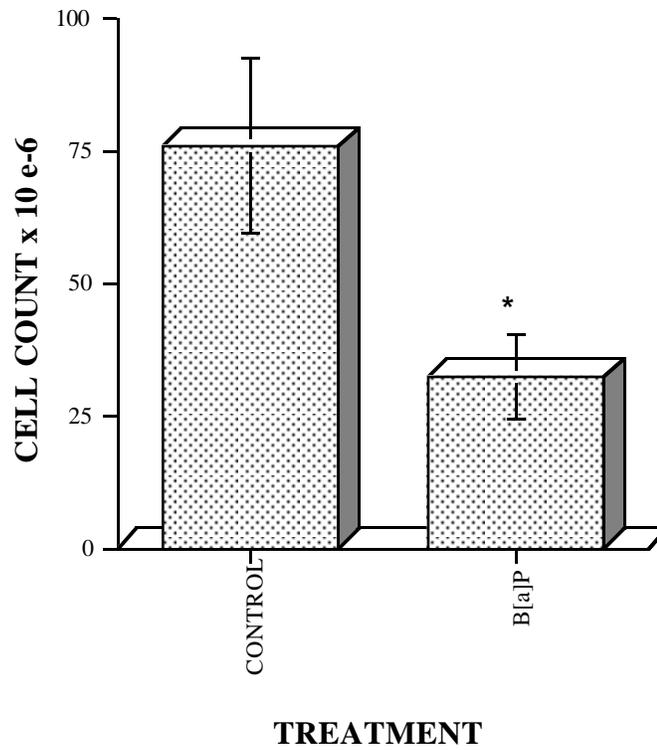


FIGURE 3

Effect of B[a]P on the humoral immune response to SRBC as indicated by plaque formation using the hemolytic PFC assay

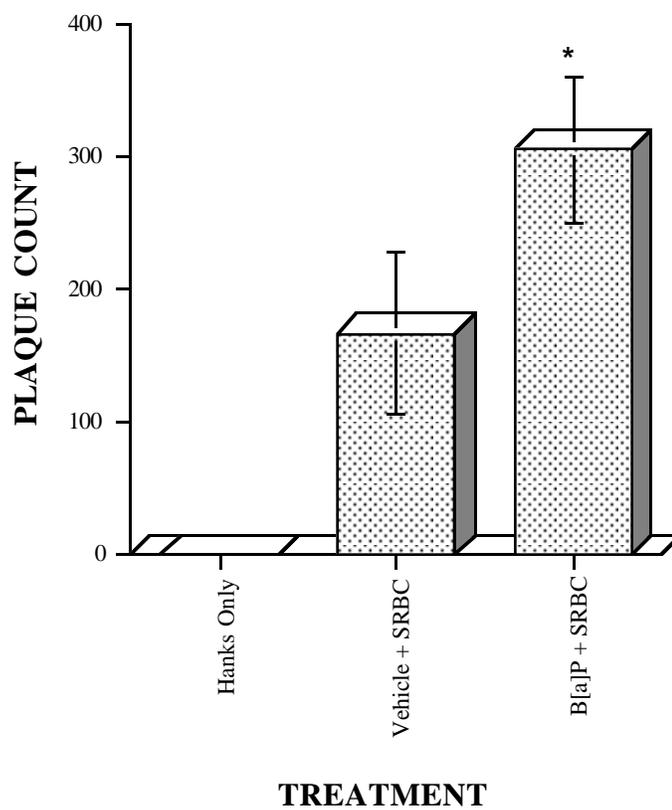
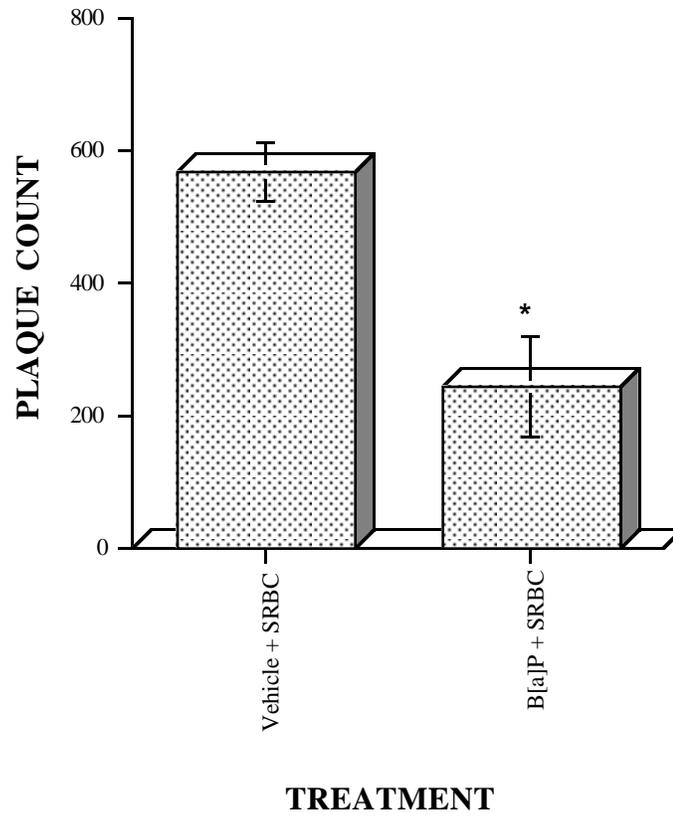


FIGURE 4

Effect of B[a]P on the humoral immune response to SRBC as indicated by plaque formation using the hemolytic PFC assay



MANUSCRIPT II*: *Tilapia (Oreochromis niloticus)* and Rodents Exhibit Similar Patterns of Antibody Production Following Exposure to Chemical Inhibitors of Humoral Immune Function

Smith DA, Schurig GG, Smith SA, and Holladay, SD

(*Submitted to *Toxicological Sciences*)

ABSTRACT

The hemolytic plaque forming cell assay (PFC), a measure of ability to produce specific antibody following challenge with antigen, has been found to be a powerful predictor of immunosuppression in chemical-exposed rodents. The efficacy of this assay for predicting humoral immunosuppression in non-rodent species remains unknown, however. In the present report, tilapia (*Oreochromis niloticus*) were exposed to nine chemical agents known to inhibit antibody production in mice (benzo[a]pyrene (B[a]P), 7,12-dimethylbenzanthracene (DMBA), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), dimethylnitrosamine (DMN), cadmium chloride (CdCl₂), azathioprine (AZA), T₂ mycotoxin (T₂-toxin), hexachlorocyclohexane (lindane, HCH- γ), and toluene), and five chemical agents which do not inhibit this response in mice (oxymethalone, acetonitrile, diethylstilbesterol (DES), tert-butylhydroquinone (TBHQ), and formaldehyde). Eight of the nine agents which inhibit rodent humoral immune responses also caused decreased PFC responses in tilapia. All five of the compounds with negative humoral effects in rodents were also negative in fish. Thus, 13 of the 14 chemical agents tested gave similar results

in fish as reported in rodents, indicating a comparable pattern of humoral immunosuppression in chemical-exposed tilapia to that seen in laboratory rodent models.

Key Words: Tilapia, fish, humoral immunity, plaque forming cell assay (PFC), risk assessment

INTRODUCTION

Considerable interest has evolved over the past approximately eight to ten years regarding interrelationships between environmental contaminants, immune impairment, and health/disease in fish (Anderson, 1990; Kelly et al., 1993; Wester et al., 1994; Weeks et al., 1990, 1986). In this regard, sediment-bound environmental contaminants have been associated with a variety of external and internal lesions in fish, which appear to be related to chemical-induced suppression of fish immune function (Anderson, 1990; Wolf, 1988; and Dean et al., 1994). Immunosuppression related to similar chemical exposure has additionally been implicated in outbreaks of infectious diseases in both commercial and wild fish populations, culminating in significant economic loss (Wester et al., 1994; Luster et al., 1988). Thus, immune dysfunction resulting from pollution of waters important to aquaculture by industrial, agricultural, and domestic wastes is a real problem, which may contribute to decreased fish productivity (Wolf, 1988; Dean et al., 1994; and Luster et al., 1992). Further, the

increased incidence of tumors and immune-complex related lesions in these fish provide direct evidence that pollutant-mediated inhibition of immune function may be decreasing fish yield (Anderson, 1990; Luster et al., 1992; and Holladay et al., 1998). Contributing to the critical nature of these observations, it is now realized that such decreased production may extend to fish hatcheries, farms, and aquaculture stations, which are becoming increasingly important in supplementing both food and sport fisheries (Wester et al., 1994; Holladay et al., 1998; and Weeks and Warinner, 1986). The latter problem may result from the fact that these facilities often make use of local open-water sources, or generally subject to the same run-off contamination by insecticides, herbicides, and halogenated/polycyclic hydrocarbons which occur in other area waters. As a result, many reports presently exist in the literature linking chemical stress with fish disease and kills (Anderson et al., 1990; Weeks et al., 1986; Luster et al., 1988; Holladay et al., 1998; and Weeks and Warinner; 1986). This problem underscores the present need for identification and development of suitable (i.e., sensitive) assays of fish immune function, which will assist aquaculturists with important decisions regarding water source quality and problem solving in facilities hampered by low production.

Traditional rodent immunotoxicity risk assessment using a tier-wise battery of immune tests has indicated that depressed antibody-mediated immunity is an especially sensitive indicator of immunotoxicant exposure (Luster et al., 1988). More recently, a database representing results of over 50

selected compounds was generated in rodents by the National Institute of Environmental Health Sciences (NIEHS) Immunotoxicology Laboratory, the National Toxicology Program-sponsored laboratories, and the Chemical Industry Institute of Toxicology (CIIT), in an attempt to provide information to aid in quantitative risk assessment for immunotoxicity (Luster et al., 1992). The two tests indentified by this work as the most predictive for actual immunosuppression were the antibody plaque forming cell assay (PFC), and splenic T and B lymphocyte quantitation using monoclonal antibodies. The latter of these tests presently cannot be conducted in most fish species, including tilapia, due to a lack of specific monoclonal antibody reagents. However, we have recently developed and then tested a modified PFC in tilapia exposed to the immunotoxicant benzo[a]pyrene (Smith et al., manuscript submitted). The focus of the present study is to extend this previous work by evaluating the effect of nine chemically-diverse rodent immunotoxicants and five non-rodent immunotoxicants with the PFC in tilapia, and thus, to determine if inhibited antibody production may serve as a sensitive indicator of immunotoxicant exposure in fish.

MATERIALS AND METHODS

ANIMALS. Tilapia were produced in-house at the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine

(VMRCVM) and at the Aquaculture Center of Virginia Polytechnic Institute and State University. Fish of approximately the same age and average weights of 142.7 ± 11.3 g were arbitrarily selected, then transferred individually to three separate and equal compartments in 80 L long tanks with filtered, aerated, and dechlorinated water. Temperature and lighting conditions were maintained at 26 °C with a 12/12 hour light/dark cycle. Ammonia and nitrite levels were monitored weekly, and tanks were cleaned as needed. Fish were fed a commercial fish diet of floating nuggets or brood stock pellets (Zeigler Bros., Inc., Gardners, PA) at 2% body weight every other day, and were allowed a two-week acclimation period before each experiment.

REAGENTS. Hank's buffered saline solution (HBSS) was prepared fresh (Hudson and Hay, 1989) and adjusted to pH 7.3 with either 1N HCl or 1N NaOH, then filter sterilized. Complete media was prepared fresh with RPMI 1640 containing hepes (25 mmol) and L-glutamine (2 mmol) (Mediatech, Fisher Scientific, Fair Lawn, NJ) with the addition of 50 IU/mL penicillin (ICN, Costa Mesa, CA), 50 mg/mL streptomycin (ICN) and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Trypan blue (Flow Laboratories, Fisher Scientific) used for differentiation, enumeration, and viability assessment of lymphocytes was diluted 1:10 in phosphate buffered saline (PBS, Sigma Chemical Co., St. Louis, MO). Bacteriological Agar (Sigma) at 1.4% was used for creating a level surface for antibody production in 5 cm petri dishes (Fisher). Bacterial agar at

0.7% was used for suspension of lymphocytes and SRBC (Hudson and Hay, 1989). Diethylaminoethyl-dextran (DEAE, Sigma) was used to avoid DNA perturbation in the 0.7% agar suspension. Tricaine methanesulfonate (MS-222, Sigma) was used in instances where fish anesthesia was necessary at a concentration of 1.2 g per 6.0 L of water.

CHEMICALS AND DOSING. Dosing solutions were prepared as follows: B[a]P, DMBA, TCDD, DMN, DES, lindane, oxymethalone, toluene, and acetonitrile (Sigma) were dissolved in corn oil with gentle heat at approximately 60 °C with periodic stirring until completely dissolved. T₂-toxin (Sigma) was dissolved in 200 µL EtOH, then added to corn oil. CdCl (Sigma) and TBHQ (Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in H₂O at room temperature. Azathioprine (Sigma) was dissolved in NaOH and isotonic saline as previously reported (Smith et al., submitted). Formaldehyde (Sigma) was diluted to 40% in distilled H₂O.

Sheep blood was obtained from one of two sheep housed VMRCVM in 500 mL sterile bottles with sodium citrate added as an anticoagulant. Fifteen milliliters of sheep blood were washed in HBSS three times at 400 × g, 10 min, 4 °C, and the resulting pellet of SRBC was diluted to 20% in HBSS and stored at 4 °C.

All chemical dilutions were prepared at a low-dose range, i.e., that which would not cause overt signs of toxicity. Dosings were delivered via

intraperitoneal injection using tuberculin syringes and 25-gauge needles, with the exception of formaldehyde, which was added directly to aquarium water. Chemical dilutions and dosages are listed in Table 1.

Two sets of three fish each were sampled (6 total, N=3 per dosing group) per chemical in duplicate. All six fish within each chemical set (with the exception of formaldehyde-dosed fish) were injected with 0.2 mL/100 g body weight of 20% SRBC on day 1 and day 14 to assess the secondary response to the SRBC immunogen. Chemical and vehicle control exposure occurred on day 7, with three fish receiving chemical, and three receiving vehicle control. The immunopoietic pronephros (anterior kidney) tissue was sampled on day 21 (seven days post-secondary immunization) (Table 2).

CELL PREPARATION AND ENUMERATION. Anesthetized fish were sacrificed via cranial blow, weighed, and placed on ice. The pronephros tissue was excised under sterile conditions and immediately placed into 5 mL of cold HBSS in sterile plastic 7 cm petri dishes (Fisher). Pronephric cells were separated by gentle maceration over 60 μ m autoclavable wire mesh screening, transferred to 15 mL conical tubes (Fisher), and washed one time in cold HBSS at 400 x g, 7 min, 4 °C. The resulting cellular pellet was resuspended in 4 mL cold complete media and placed on ice. A 50 μ L aliquot was removed and combined with 450 μ L 10% trypan blue for lymphocyte differentiation,

enumeration and viability in a hemacytometer at 40X. Each fish sample was diluted to 40.0×10^6 lymphocytes per mL in complete media, and stored on ice.

COMPLEMENT PREPARATION. Homologous tilapia blood serum was used as a compatible complement source. For each experiment, two large tilapia (approximately 500 g) were anesthetized using MS-222 and aseptically bled via caudal venal puncture, obtaining a total volume of 3 mL per fish. The blood was immediately transferred to two 3 mL red-topped glass Vacutainers® without additives and the serum was allowed to clot at room temperature for 45 min. The clots were not allowed to adhere to the sides of the tubes. The tubes were then transferred to ice for one hr to facilitate complete serum separation, and centrifuged at $400 \times g$, 7 min, 4°C . Serum was collected via Pasteur pipette, diluted to 10% in cold HBSS, and stored on ice for no longer than 30 min before use to avoid denaturation of the complement components.

AGAR PREPARATION. A 0.7% solution of agar was prepared by combining 0.35 g of agar plus 0.025 g of DEAE with 50 mL HBSS at 80°C with gentle stirring until just dissolved, then transferred to a 45°C water bath. Assay procedures were conducted quickly at this point to avoid agar gel precipitation.

PFC ASSAY and ENUMERATION. Two-hundred and fifty microliters of each serum sample were combined with 0.15 mL 20% SRBC and 0.80 mL of 45°C

0.7% agar/DEAE solution, mixed quickly, then poured into 5 cm plastic petri dishes and allowed to solidify. Plates were incubated for two hrs with humidity at 30 °C to allow specific lymphocyte recognition of SRBC and subsequent antibody production and binding. One milliliter of the 10% complement source serum was then added, and the plates were returned to incubation overnight to allow complement mediated-lysis of the SRBC (plaque formation). Plaques were enumerated and evaluated for approximate size manually via low-powered microscopy using a Nikon AFX-DX dissecting microscope with a 10X ocular strength.

STATISTICAL ANALYSIS. Experiments were designed under normal analysis of variance parameters (Sokal and Rohlf, 1995) and were analyzed using a SAS computer analysis package designed especially for this application, employing cross-wise comparisons using Tukey's and Student's t-analyses, and a significance level of $P < 0.05$.

RESULTS

All fish which received vehicle and SRBC (positive controls) presented as clinically normal throughout the dosing regimens. Gross examinations of the pronephros tissue of the positive controls showed numerous darkly pigmented

spherical bodies and an overall brownish color as reported by previous SRBC immunization studies in fish (Sailendri and Muthukkaruppan, 1975).

Dose range-finding studies were conducted in fish to determine levels producing overt toxicity (increased mortality; abnormal swimming behavior; temporary loss of equilibrium). No more than 50% of an overtly toxic level of chemical exposure was used in experiments monitoring humoral immune function. Transient limited toxicity in fish (hyperpigmentation of skin and fins, slight lethargy, and temporary reduction of feed intake) were observed after exposure to some of the selected chemical agents.

The pronephric tissue of the vehicle-exposed fish and fish dosed with oxymethalone, toluene, acetonitrile and TBHQ appeared dark reddish in color (clinically normal), with numerous pigmented bodies. The pronephric tissue of the fish dosed with azathioprine, B[a]P, DMBA, TCDD, DMN, CdCl, DES and T₂ typically was more friable and appeared dark brownish in color, with numerous pigmented bodies similar to that seen in the positive control fish.

PLAQUE ENUMERATION. Fish dosed with azathioprine, B[a]P, DMBA, DMN, CdCl, T₂-toxin, TCDD, or toluene displayed significantly reduced plaque numbers compared to the vehicle dosed fish (Figures 1-8). Fish exposed to lindane, acetonitrile, DES, oxymethalone, formaldehyde or TBHQ showed no significant difference in plaque formation compared to vehicle-dosed controls (Figures 9-14).

DISCUSSION

The PFC was initially developed by Jerne in 1961, and has since become a mainstay of routine cellular immunology research, primarily accessing humoral IgM antibody responses to antigen (Ostrander, 1996). Briefly, specific B and T lineage lymphocyte populations previously presented *in vivo* with heterologous erythrocytes are agar plated in combination with the identical erythrocyte suspensions used for immunization. During incubation, the B-cells secrete IgM antibody to antigenic determinants present on the surface of the erythrocytes (most commonly with T-cell help), resulting in antibody-erythrocyte binding. The presence of an adequate complement source allows complement-mediated lysis of the antibody-coated erythrocytes, resulting in the formation of clear zones or “plaques” in the agar. Located within the center of each plaque is a single, antibody producing plasma cell. As a lytic site can be produced by a single IgM molecule fixing one C1q complement molecule, high sensitivity is a major advantage of the PFC assay.

The PFC response requires coordinated interactions of antigen presenting cells, regulatory T cells, and B cells. Any compound-induced alteration in antigen processing, presentation, cell proliferation and/or differentiation, or related synthesis and/or release of interleukins, could modify this response (Luster et al., 1992). As a result, the antibody PFC response to SRBC appears to be the most commonly affected immune functional parameter in animals

exposed to chemical immunosuppressants (Luster et al., 1988). Further, this test has a calculated individual predictive value for immunosuppression in B6C3F1 mice of 0.78 (i.e., a chemical-induced significant depression of the PFC response was accompanied by clinically-significant immunosuppression approximately 80% of the time). These results raise questions regarding the efficacy of the antibody PFC response for evaluating immune function in non-rodent species (e.g., fish) exposed to chemical immunotoxicants.

The database generated by the National Toxicology Program for immunotoxicity risk assessment in rodents was based on compiled data from studies using over 50 selected compounds (Luster et al., 1992). These compounds were given an immunotoxicity classification (positive or negative) by the NTP based on host resistance studies (challenge assays including resistance to bacterial or viral pathogens, parasitic agents, or syngeneic tumor cells). For the present studies, nine chemical agents classified as humorally immunotoxic in rodents, and five agents classified as non-humorally immunotoxic, were evaluated in fish. The immunotoxic agents studied in fish were purposefully selected as chemically-diverse, and included two polycyclic aromatic hydrocarbons (B[a]P and DMBA), a halogenated aromatic hydrocarbon (TCDD), a chlorinated insecticide (lindane), a heavy metal (CdCl₂), an alkylating agent (DMN), a human antineoplastic/therapeutic immunosuppressant (AZA), and an immunosuppressive fungal mycotoxin (T₂).

Of these immunotoxic agents tested for ability to depress humoral immune function in fish, all but one (lindane) significantly reduced antibody production. Thus, with the exception of lindane, responses in the present chemical-exposed fish compared well with rodents. Lindane is a well-established immunotoxicant, and does depress both the primary and secondary antibody responses to SRBC in mice (Banerjee et al., 1996). We have previously reported hypocellularity of the pronephros and spleen of tilapia exposed to lindane (Hart et al., 1997), thus lindane appears to produce immunotoxicity in these fish. The lack of a depressed PFC response in the tilapia PFC assay may be the result of the dose and/or dosing schedule used (a single acute exposure), or it may be that, for undetermined reasons, lindane does not affect fish humoral immune function in a manner comparable to rodents. In this regard, chronic lindane exposure in carp (*Yersinia ruckeri*) did not inhibit antibody production (Cossarini-Dunier et al., 1987), thus it is possible that humoral immune function in mice is more sensitive to lindane than in fish. Further testing will be required to make this determination.

When the PFC test was conducted in tilapia treated with chemical agents classified in the 1992 NTP report as non-immunotoxic, one of the chemical agents (toluene) caused a significant reduction in plaque numbers in treated fish. However, experimental results published subsequent to the NTP-sponsored studies have demonstrated immunotoxicity in several species exposed to toluene. Cheremisinoff et al. (1994) found that toluene was

genotoxic to bone marrow hematopoietic cells, causing decreased peripheral lymphocyte counts in mice, rats, rabbits, cows, non-human primates, and humans. Mice exposed to toluene vapors exhibited increased susceptibility to respiratory infection with *Streptococcus zooepidemicus* and decreased bacteriocidal activity against *Klebsiella pneumoniae* (Klaassen, 1996). Additionally, mice dosed with toluene in their drinking water were found to have inhibited T and B cell lymphoproliferative responses, thymic involution, and an inhibited PFC response (Smialowicz and Holsapple, 1996). Thus, although we were unaware of such at the time of experimentation in fish, toluene is presently recognized as a rodent immunotoxicant. Most relevant to the present study, toluene had also been found to depress rodent antibody production.

In summary, the PFC assay is an established, powerful, individual test of chemical-induced immunosuppression in rodents. As such, this assay is commonly used as a sensitive indicator of rodent immunotoxicant exposure. We therefore evaluated the PFC assay in tilapia exposed to chemically-diverse immunotoxic and non-immunotoxic compounds to determine if antibody production in fish was generally sensitive to known rodent immunotoxicants, and to compare the fish outcome to the published rodent data. Considering that toluene is now recognized as an immunotoxic compound which inhibits PFC responses in mice, 8 of 9 chemical agents known to reduce antibody production in mice also caused this effect in fish (lindane being the exception).

Five of five non-humorally immunotoxic chemicals in mice also tested as non-immunotoxic in fish. Thus, 13 of the 14 chemical agents gave similar results in fish as reported in rodents, indicating that the humoral immune response of chemical-exposed tilapia compares well with rodents.

The present results with the PFC assay in fish suggest that it may be possible to develop other standard rodent immunotoxicity assays for use in fish, including the cytotoxic T-lymphocyte assay (CTL). Specifically, the CTL and PFC assays in B6C3F1 mice presently have an estimated pairwise predictive value for immunosuppression of 1.0 (i.e., to date, all chemical agents which suppress both of these assays in the mouse model also produce immunosuppression). It would be interesting and important to determine if these two assays have similar high pairwise concordance for predicting immunosuppression in fish.

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TABLES AND FIGURES

Table 1: Dosage dilutions and amounts of chemicals and vehicles used for the PFC assay in tilapia.

Table 2: Dosing schedule followed for all 14 chemicals tested in the tilapian PFC assay, including SRBC and vehicles.

Figure 1: Effects of azathioprine on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 10.0 mg/kg AZA on experimental day 7. Vehicle exposed fish received saline on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in AZA treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.0084$.

Figure 2: Effects of B[a]P on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 5.0 mg/kg B[a]P on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque

formation was observed in B[a]P treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.0031$.

Figure 3: Effects of DMBA on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 5.0 mg/kg DMBA on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in DMBA treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.0005$.

Figure 4: Effects of DMN on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 1.0 mg/kg DMN on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in DMN treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.0014$.

Figure 5: Effects of CdCl on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 1.0 mg/kg CdCl on experimental day 7. Vehicle exposed

fish received distilled water on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in CdCl treated fish. * indicates significantly different from the vehicle + SRBC dosed group, P=0.0028.

Figure 6: Effects of T₂ on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 0.1 mg/kg T₂ on experimental day 7. Vehicle exposed fish received corn oil plus EtOH on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in T₂ treated fish. * indicates significantly different from the vehicle + SRBC dosed group, P=0.0028.

Figure 7: Effects of TCDD on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 1.0 mg/kg TCDD on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in TCDD treated fish. * indicates significantly different from the vehicle + SRBC dosed group, P=0.0009.

Figure 8: Effects of toluene on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 0.5 mg/kg toluene on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. Reduced plaque formation was observed in toluene treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.0007$.

Figure 9: Effects of lindane on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 20.0 mg/kg CdCl on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. No reduction of plaque formation was observed in lindane treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.1370$.

Figure 10: Effects of acetonitrile on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 10.0 mg/kg acetonitrile on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. No reduction of

plaque formation was observed in acetonitrile treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.5327$.

Figure 11: Effects of DES on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 6.0 mg/kg DES on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. No reduction of plaque formation was observed in DES treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.9551$.

Figure 12: Effects of oxymethalone on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 1.0 mg/kg oxymethalone on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. No reduction of plaque formation was observed in oxymethalone treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.6171$.

Figure 13: Effects of formaldehyde on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 100 ppm formaldehyde in 20 gal of water for 30 minutes

on experimental day 7. Vehicle exposed fish were placed in 20 gal of water without formaldehyde for 30 minutes. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. No reduction of plaque formation was observed in formaldehyde treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.5353$.

Figure 14: Effects of TBHQ on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay. Chemical-exposed fish received 10.0 mg/kg TBHQ on experimental day 7. Vehicle exposed fish received distilled water on experimental day 7. All fish received SRBC at 0.2 mL/100g body weight on experimental days 1 and 14. No reduction of plaque formation was observed in THBQ treated fish. * indicates significantly different from the vehicle + SRBC dosed group, $P=0.0793$.

TABLE 1

**Dosage dilutions and amounts of chemicals and vehicles
used for the PFC assay in tilapia**

Chemical	Dilution	Vehicle	Average Dosage Amount
Acetonitrile	10.0 mg/kg	dWater	0.45 mL
AZA	10.0 mg/kg	Saline	0.70 mL
B[a]P	5.0 mg/kg	Corn Oil	0.15 mL
CdCl	1.0 mg/kg	dWater	0.45 mL
DES	6.0 mg/kg	Corn Oil	0.25 mL
DMBA	5.0 mg/kg	Corn Oil	0.30 mL
DMN	1.0 mg/kg	Corn Oil	0.50 mL
Formaldehyde	100 ppm	Water	8 mL/20 gal
Lindane	20.0 mg/kg	Corn Oil	0.55 mL
Oxymethalone	1.0 mg/kg	Corn Oil	0.50 mL
T ₂	0.1 mg/kg	Corn Oil + ETOH	0.15 mL
TBHQ	10.0 mg/kg	dWater	0.45 mL
TCDD	1.0 mg/kg	Corn Oil	0.35 mL
Toluene	0.5 mg/kg	Corn Oil	0.35 mL

TABLE 2

**Dosing schedule followed for all 14 chemicals tested in the tilapian PFC assay,
including SRBC and vehicles**

<u>Fish Number</u>	<u>Day 1</u>	<u>Day 7</u>	<u>Day 14</u>	<u>Day 21</u>
1, 2, 3	SRBC	Vehicle	SRBC	Sampled
4, 5, 6	SRBC	Chemical	SRBC	Sampled

FIGURE 1

Effects of Azathioprine on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

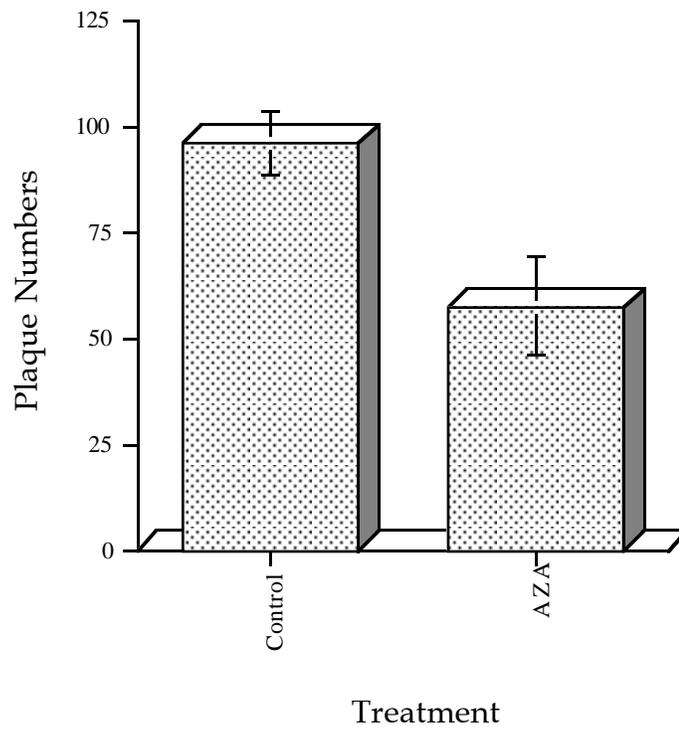


FIGURE 2

Effects of B[a]P on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

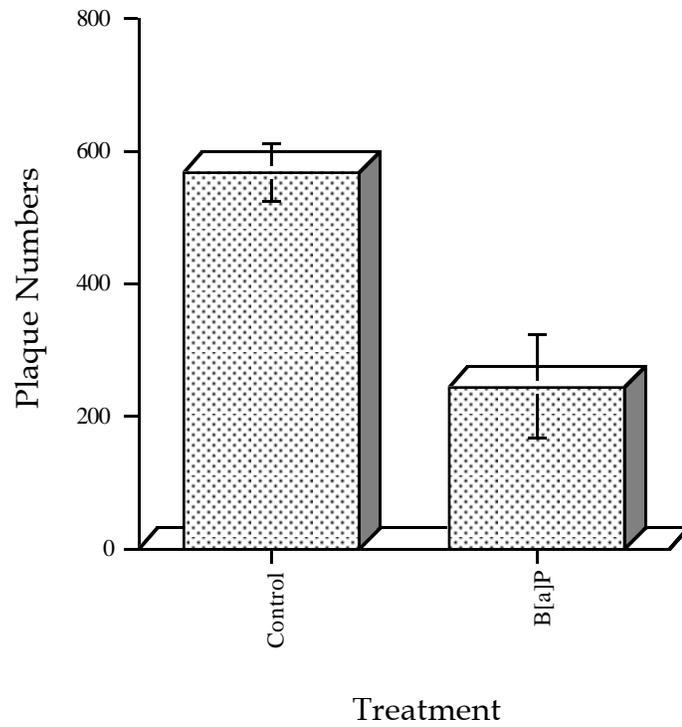


FIGURE 3

Effects of DMBA on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

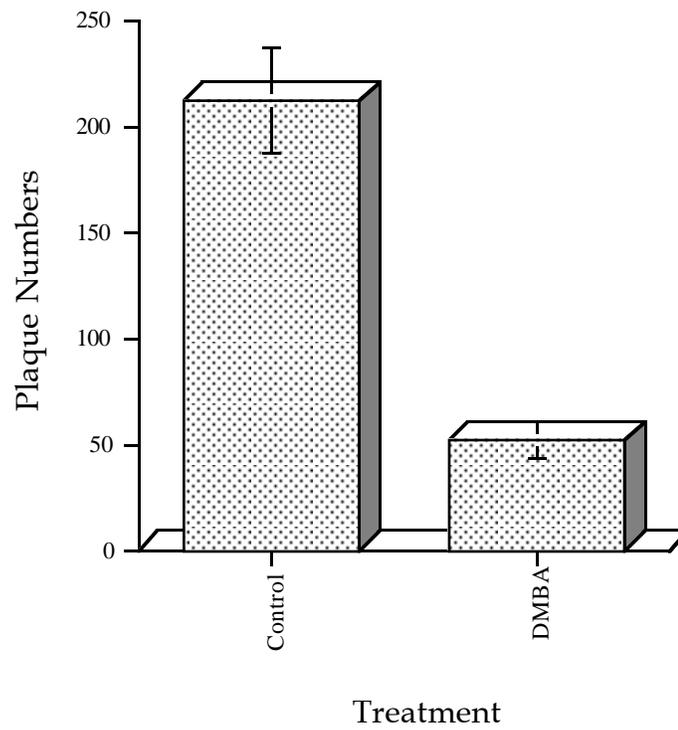


FIGURE 4

Effects of DMN on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

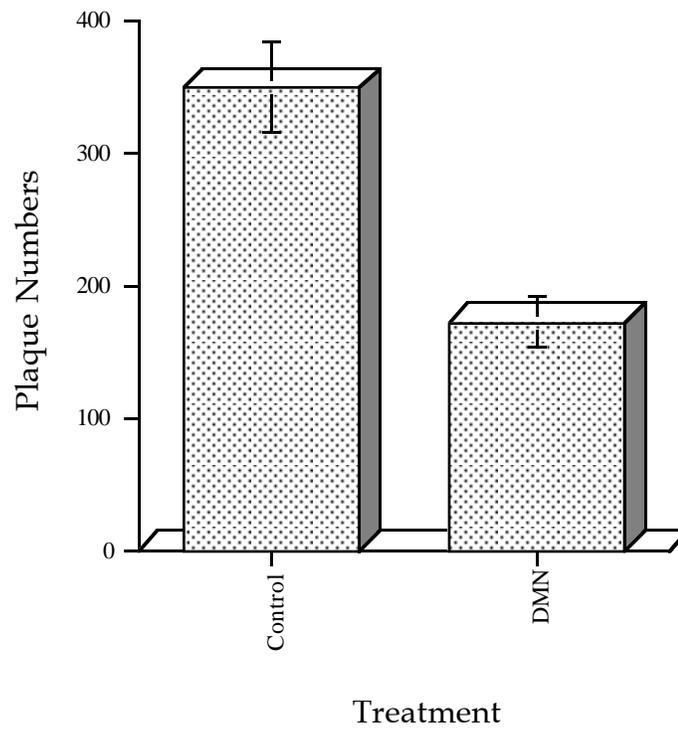


FIGURE 5

Effects of CdCl on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

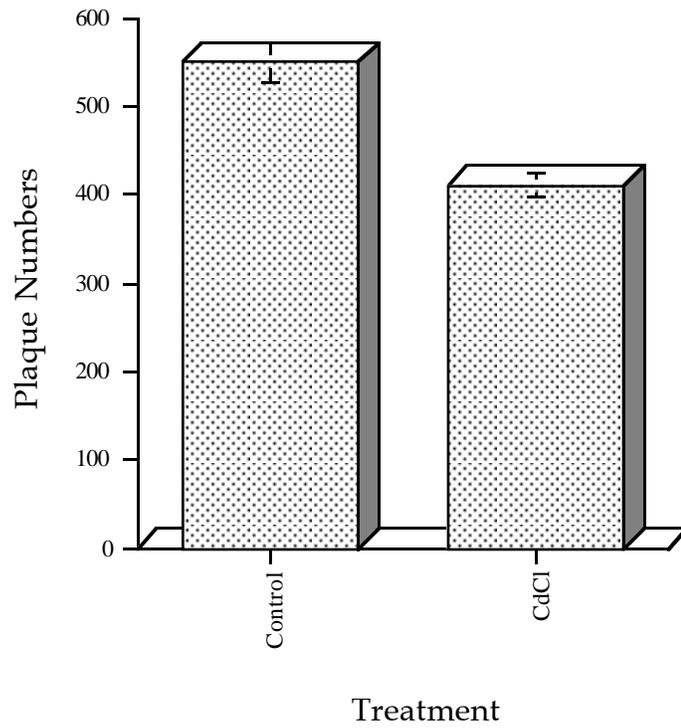


FIGURE 6

Effects of T₂-toxin on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

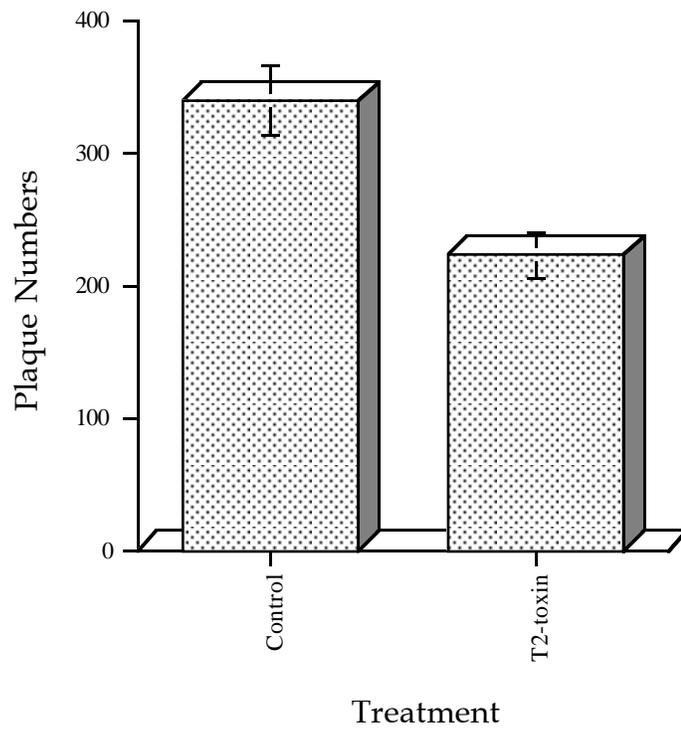


FIGURE 7

Effects of TCDD on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

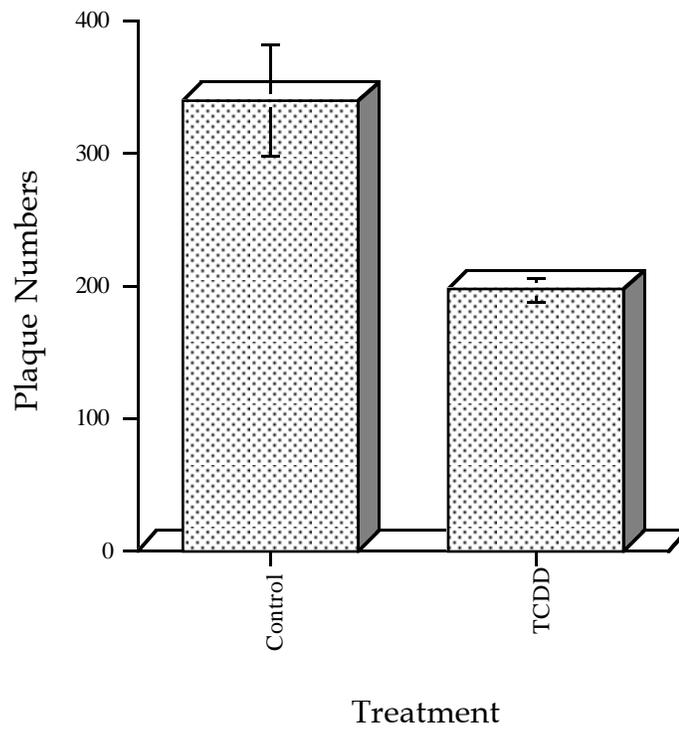


FIGURE 8

Effects of toluene on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

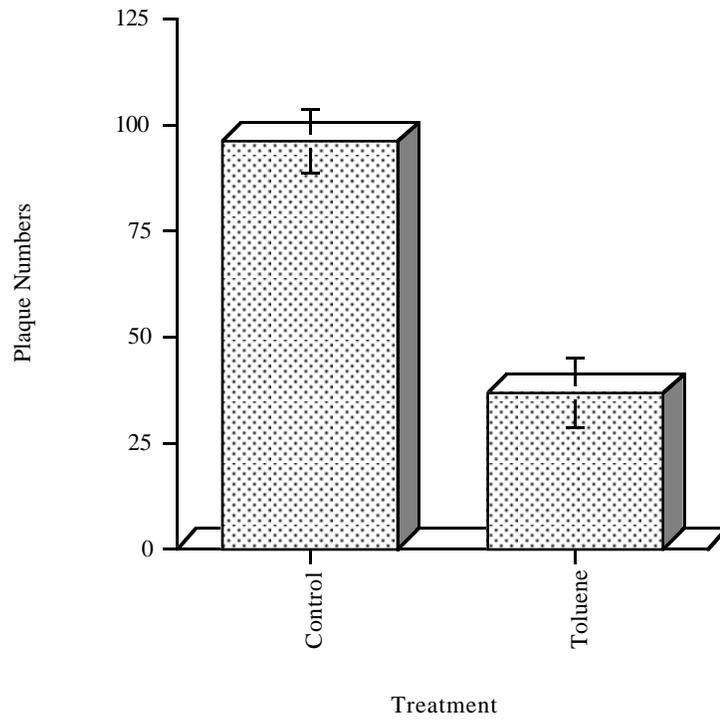


FIGURE 9

Effects of lindane on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

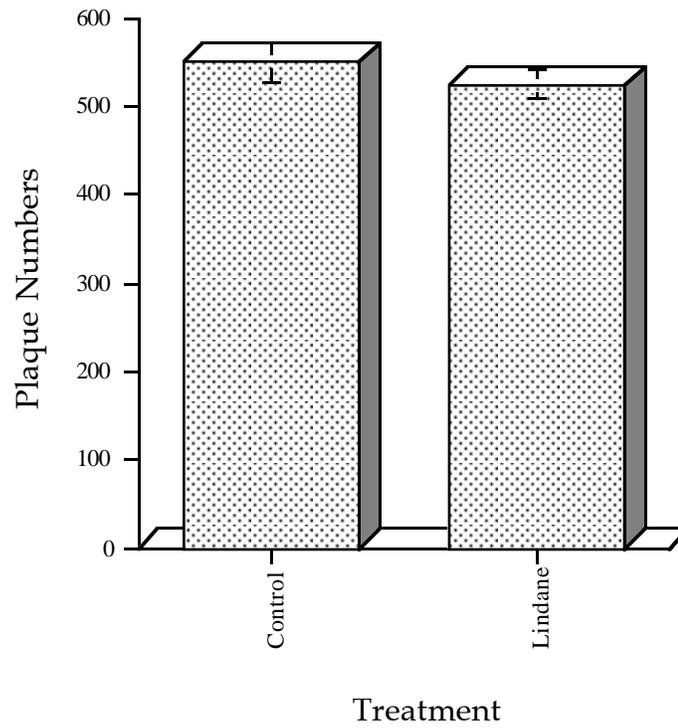


FIGURE 10

Effects of acetonitrile on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

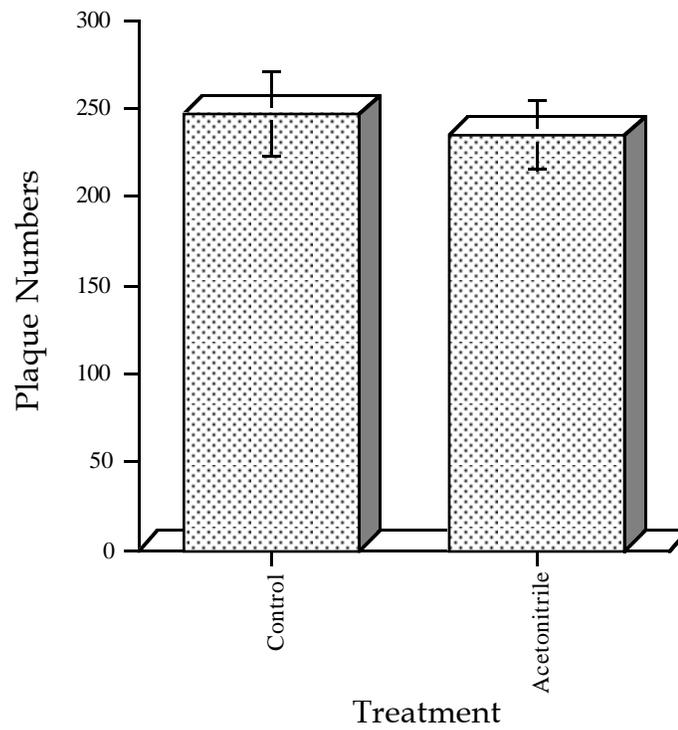


FIGURE 11

Effects of DES on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

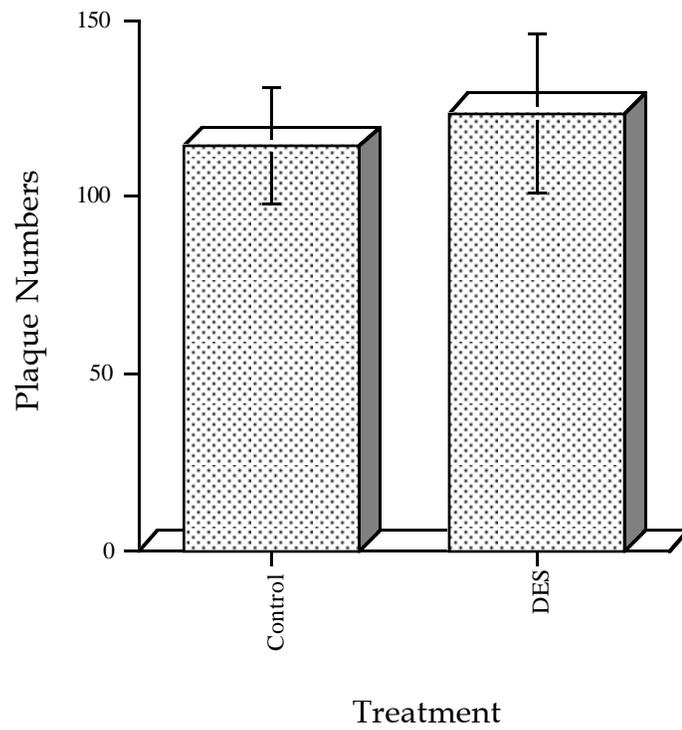


FIGURE 12

Effects of oxymethalone on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

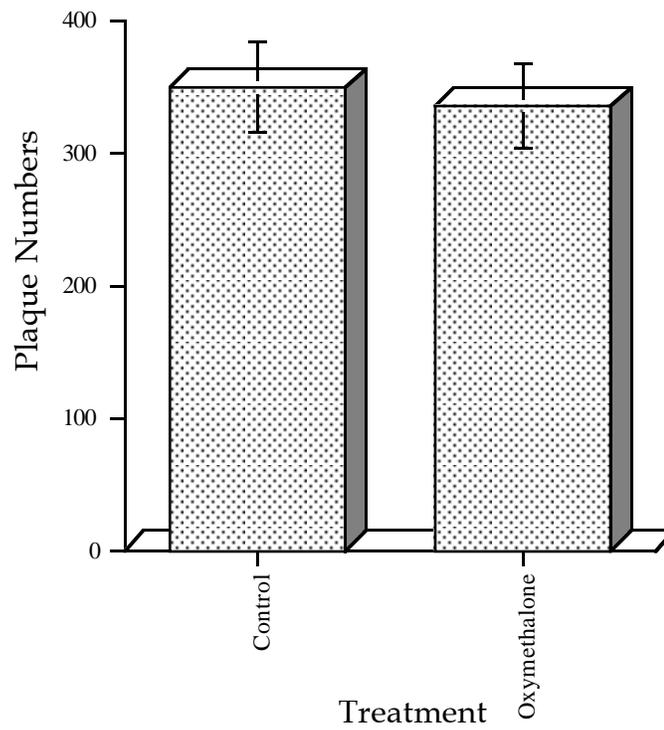


FIGURE 13

Effects of formaldehyde on the tilapian humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.

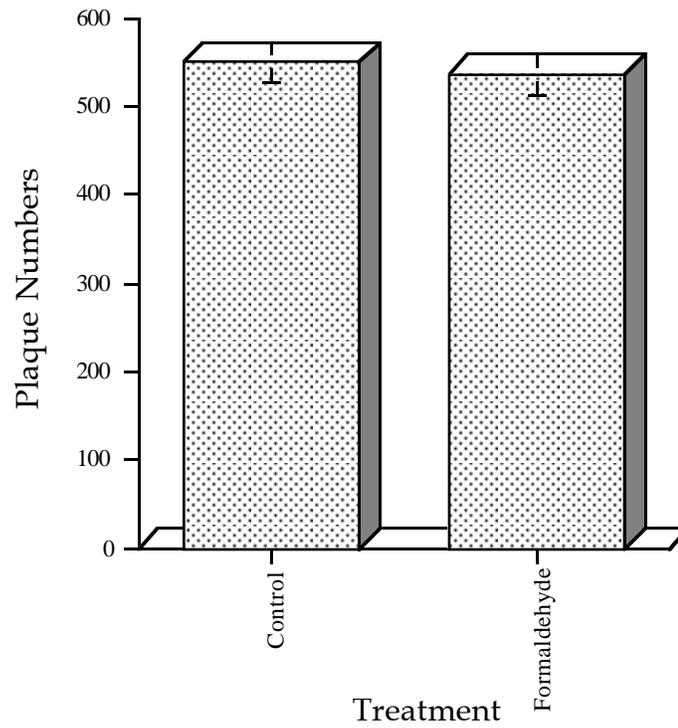
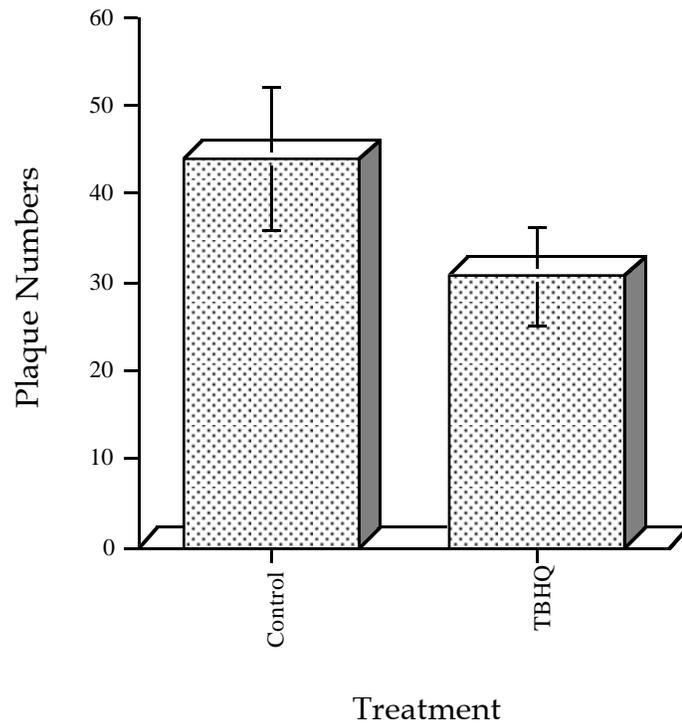


FIGURE 14

Effects of TBHQ on the tilapia humoral response to SRBC as indicated by plaque formation using the hemolytic PFC assay.



MANUSCRIPT III: The Development and Application of a Cytotoxic T-Lymphocyte Assay (CTL) in Tilapia (*Oreochromis niloticus*) for Immunotoxicity Risk Assessment Yields Results Similar to that of the Rodent Model

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Abstract

The cytotoxic T-lymphocyte (CTL) assay, a measure of the ability of cytotoxic T-cells to target and lyse cells expressing foreign antigen, has been found to be a powerful individual predictor of inhibited immune function in chemical-exposed rodents. However, the efficacy of this assay for predicting immunosuppression in non-rodent species remains unknown. In the present report, tilapia (*Oreochromis niloticus*) were exposed to 10 chemical agents known to inhibit CTL activity in mice (benzo[a]pyrene, 7,12-dimethylbenzanthracene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, dimethylnitrosamine, cadmium chloride, azathioprine, T₂ mycotoxin, hexachlorocyclohexane, diethylstilbesterol, and toluene), and four chemical agents which do not inhibit this response (oxymethalone, acetonitrile, tert-butylhydroquinone, and formaldehyde). Nine of the ten agents known to inhibit rodent CTL responses also caused decreased CTL responses in fish. All four of the compounds with negative CTL effects in rodents were also negative in fish. Thus, 13 of the 14 chemical agents tested gave similar results in fish as reported in rodents, indicating a comparable

pattern of T-cell mediated immunosuppression in chemical-exposed tilapia to that seen in the laboratory rodent models.

Key Words: Tilapia, fish, humoral immunity, cytotoxic T-lymphocyte assay (CTL), risk assessment

Introduction

Immunotoxicology has become an area of major interest in the last decade. This has been stimulated by increasing knowledge of immunology and the importance of the immune response in maintaining the integrity of the organism. In particular, considerable interest has recently evolved regarding interrelationships between environmental contaminants, immune impairment, and health and disease in fish (Seeley and Weeks-Perkins, 1991; MacCubbin et al., 1990; Anderson, 1990; Kelly et al., 1993; and Wester et al., 1994). The focus of the present research is two-fold: 1) To begin to characterize in a controlled laboratory setting the effect of a chemically-diverse group of known rodent immunotoxicants on fish immune function, and 2) To evaluate the use of traditional rodent immunotoxicity risk assessment methodologies in fish as possible sensitive indicators of immune impairment. Providing this information will assist in aquaculture industry decisions regarding minimal water quality standards necessary for maintaining fish health, as well as in delineation of production problems related to water/sediment contamination

by immunosuppressive chemicals. Such has become necessary due to widespread contamination of the aquatic environment with immunosuppressive pollutants, including (organo)metals, halogenated hydrocarbons, heterocyclic compounds, carbamates, and organophosphates (Seeley and Weeks-Perkins, 1991; Kelly et al., 1993; and Wong et al., 1992). Indeed, an increasing number of epizootics of disease in wild fish populations have been reported during the past decade in areas with a history of contamination with such chemical agents (MacCubbin et al., 1990; Couch and Harshbarger, 1985; Dean et al., 1983; and Urso and Johnson, 1987).

Evolution of immunotoxicity testing procedures in laboratory rodents led to the use of batteries of immune tests arranged in tiers for immunotoxic compound determination, a labor-intensive procedure (Vos et al., 1989; Purtilo and Linger, 1983; Koller and Exon, 1985; Murray et al., 1985; Luster et al., 1988; and Sjoblad, 1988). Assimilation of experimental rodent data collected over the past 10 years by the National Toxicology Program (NTP) has, however, provided a database which may be useful for improving the accuracy and efficiency of screening chemicals for immunotoxicity (Luster et al., 1992). Specifically, the performance of only two or three immune tests was found to be sufficient to predict immunotoxic compounds in mice (>90% concordance). Tests showing high individual association with immunotoxicity included the splenic antibody plaque forming cell (PFC) response (78%) and the cytotoxic T-lymphocyte (CTL) assay (67%). Further, the combination of inhibited CTL

cytolytic function and inhibited antibody production was found to be 100% predictive for immunosuppression. Thus, these results provided important new information regarding the ability of presently used immune tests to predict xenobiotic immunosuppressive potential. Whether certain of the above immune tests may be predictive for immunotoxicity in chemical-exposed fish has not been determined, however it is anticipated that similar confidence levels are likely to be obtained with other species once such data are available (Koller and Exon, 1985; and Luster et al., 1992).

We recently evaluated the PFC assay in tilapia exposed to nine immunotoxic and five non-immunotoxic compounds, to determine if antibody production in fish was generally sensitive to known rodent immunotoxicants (i.e., to compare the fish outcome to the published rodent data). Eight of nine chemical agents known to reduce antibody production in mice also caused this effect in fish (hexachlorocyclohexane, or lindane, being the exception) (Smith et al., submitted). Five of five non-immunotoxic chemicals in mice also tested as non-immunotoxic in fish. Thus, 13 of 14 chemical agents gave similar results in fish as reported in rodents, indicating the humoral immune response of chemical-exposed fish may compare well with rodents. Given that the rodent CTL assay has been found to be a strong individual predictor of rodent immuno-suppression, and given that the CTL assay has a particularly strong pairwise predictive value for immunosuppression when combined with the PFC assay, the present report extends our previous studies by evaluating the

ability of the cytotoxic T-lymphocyte assay to detect immunotoxic chemical exposure in fish.

Materials and Methods

Animals. Tilapia were produced in-house by the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) and by the Aquaculture Center of Virginia Polytechnic Institute and State University. Fish of approximately the same age and average weights (187.8 ± 13.5 g) were arbitrarily selected, then transferred individually to 80 L long tanks partitioned into three equal-sized compartments (three fish per tank), with filtered, aerated, and dechlorinated water. Fish used for bleeding purposes were approximately 600 g and were held separately in 150 gal round tanks. Temperature and lighting conditions for all fish were maintained at 26 °C with a 12/12 hour light/dark cycle. Ammonia and nitrite levels were monitored weekly, and tanks were cleaned as needed. Fish were fed a commercial fish diet of floating nuggets or brood stock pellets (Zeigler Bros., Inc., Gardners, PA) at 2% body weight every other day, and were allowed a two-week acclimation period before experimentation.

Reagents. Hank's buffered saline solution (HBSS) was prepared fresh (Hudson and Hay, 1989) and adjusted to pH 7.3 with either 1N HCl or 1N NaOH, then

filter sterilized. Complete media was prepared fresh with RPMI 1640 containing hepes (25 mmol) and L-glutamine (2 mmol) (Mediatech, Fisher Scientific, Fair Lawn, NJ) with the addition of 50 IU/mL penicillin (ICN, Costa Mesa, CA), 50 mg/mL streptomycin (ICN) and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Sensitization media for lymphocyte culturing was prepared by adding 1.7 mL of 1.1143 density [5×10^{-5} M] 2-mercaptoethanol (Sigma) to 500 mL of complete media. Mitomycin C was used to halt DNA replication in target cells by adding 25 mg/mL to $<1 \times 10^7$ cells.

Lipopolysaccharide and Concanvalin A (Sigma) were used as mitogens to increase target lymphocyte differentiation and ensure log-phase growth.

51 Chromium was used to label the target lymphocytes by adding $\text{Na}^{51}\text{CrO}_4$ (ICN) at a dilution of 100 mCi/ 1×10^7 cells. The detergent NP-40 (Sigma) was used at a 2% dilution to ensure total target cell lysis for maximum release determination. Trypan blue (Flow Laboratories, Fisher Scientific) used for differentiation, enumeration, and viability of lymphocytes was diluted 1:10 in phosphate buffered saline (PBS, Sigma Chemical Co., St. Louis, MO). Tricaine methanesulfonate (MS-222, Sigma) was used in instances where fish anesthesia was necessary at a concentration of 1.2 g per 6.0 L of water.

Chemicals and Dosing. Dosing solutions were prepared as follows: B[a]P, DMBA, TCDD, DMN, DES, lindane, oxymethalone, toluene, and acetonitrile (Sigma) were dissolved in corn oil with gentle heat at approximately 60 °C with

periodic stirring until completely dissolved. T₂ toxin (Sigma) was dissolved in EtOH, then corn oil was added to dilute. CdCl and TBHQ (Sigma) were dissolved in distilled H₂O at room temperature. Azathioprine (Sigma) was dissolved in NaOH, then isotonic saline. Formaldehyde (Sigma) was diluted to 40% in distilled H₂O. Sheep blood was obtained from one of two sheep housed for this purpose at the VMRCVM, and stored in 500 mL sterile bottles with sodium citrate added as an anticoagulant. Fifteen milliliters of sheep blood were washed in HBSS three times at 400 x g, 10 min, 4 °C, and the resulting pellet of SRBC was diluted to 20% in HBSS and stored at 4 °C.

Dose range-finding studies were conducted at the onset with each chemical agent. Dosing was at levels not producing signs of overt toxicity, and in no case exceeded 50% of an approximate lethal dose to 50% of the animals (i.e., LD₅₀). Dosing was delivered via intraperitoneal injection using tuberculin syringes and 25-gauge needles, with the exception of formaldehyde, which was dosed via bath exposure. Chemical dilutions and dosages are listed in Table 1.

Two sets of three fish were sampled (6 total, N=3 per dosing group) per chemical in duplicate. All six fish within each chemical set were injected with either chemical or vehicle on day 1, with three fish receiving chemical, and three receiving vehicle control. The immunopoietic pronephros (anterior kidney) tissue was sampled on day 7.

Cell Preparation and Enumeration. Anesthetized fish were sacrificed via cranial blow, weighed, and placed on ice. The pronephros tissue was excised under sterile conditions and immediately placed into 5 mL of cold HBSS in sterile plastic 7 cm petri dishes (Fisher). Pronephric cells to be used as effectors were separated by gentle maceration over 60 mm autoclavable wire mesh screening, and transferred to 15 mL conical tubes (Fisher).

Allogeneic lymphocytes from donor fish were used as target cells. Briefly, for each experiment, one large, undosed tilapia was anesthetized using MS-222 and aseptically bled via caudal venal puncture, obtaining a total blood volume of 3 mL. The blood was immediately transferred to a 10 mL green-topped glass Vacutainer® with heparin to prevent clotting.

Effector and target lymphocytes were separated from either pronephric suspensions or whole blood, respectively, using Lymphoprep® by layering cellular suspension or blood diluted to 6 mL with HBSS over 3 mL of Lymphoprep®, and centrifuging at 500 × g, 18 min, 23 °C. The resulting band of white cells was removed, and washed three times in 8 mL of HBSS. The cellular pellets were resuspended in 4 mL of cold complete media and placed on ice. A 50 mL aliquot was removed and combined with 450 mL 10% trypan blue for lymphocyte differentiation, enumeration and viability in a hemacytometer at 40X.

Mitomycin C Exposure of Targets. To ensure that the effector cells were the only responders in the assay, the target cells were exposed to Mit C in order to block DNA replication. In a 50 mL conical tube, 1 mL Mit C [25 mg/mL] was added for each 1×10^7 cells. The tube was wrapped in foil to keep out light, and incubated at 28 °C, 5% CO₂ for 45 minutes with gentle swirling every 5-10 minutes to ensure adequate chemical contact with all cells. Immediately after incubation, target cells were washed thoroughly to remove all traces of the Mit C (4X, 400 x g, 10 min, 23 °C). Cells were counted and viability assessed as previously stated, and pellets were resuspended to 0.5×10^6 cells/mL.

Sensitization of Effector Cells. In sterile 25 cm² tissue culture flasks, cells were combined at an Effector:Target (E:T) ratio of 50:1. Sensitization media was added to bring the total volume to 20 mL, and the flasks were incubated at 28 °C, 5% CO₂, 95% humidity for five days. Media nutrients were maintained by periodically adding 5 mL of fresh sensitization media to the flasks as needed to maintain a pink media color.

Target Cell Preparation. On day 3 of incubation, a new sample of target cells was obtained from the peripheral blood of the same fish used for the sensitization target cells. Cells were separated, enumerated, and assessed for viability as previously stated, and transferred to a 25 cm² tissue culture flask. LPS and ConA were added at concentrations of 2 mg/mL and 10 mg/mL

respectively per every 1×10^7 cells to ensure lymphocyte differentiation and log-phase growth. Sensitization medium was added for total volume of 20 mL, and the flasks were incubated at 28 °C, 5% CO₂, 95% humidity for two days. Medium nutrients were maintained by periodically adding 5 mL of fresh sensitization medium to the flask as needed to maintain a pink medium color.

Radioisotope Exposure of Targets. After incubation, target cells were harvested by gentle decantation, and washed one time in 20 mL of room temperature HBSS at 400 x g, 10 min, 23 °C. After enumeration and viability ensurement of >80%, cells were re-pelleted, and the excess media was removed. In a 50 mL conical tube, targets were labeled so the cytotoxicity reaction of the effectors could be quantitatively measured by adding 100 mCi of Na⁵¹CrO₄ and 20 mL FBS for every 1×10^7 cells present. Complete media was added to bring the total volume to no more than 1 mL, and the tube was loosely capped, wrapped in lead foil, and incubated at 28 °C, 5% CO₂, 95% humidity for 1.5 hrs with gentle swirling every 5-10 minutes to ensure adequate absorption of chromium. Immediately following incubation, targets were washed thoroughly but carefully to avoid cell membrane rupturing and subsequent early release of chromium (4X, 300 x g, 12 min, 23 °C), enumerated and assessed for viability, and the pellets were resuspended to 0.5×10^6 cells/mL.

Sensitized Effector Cell Preparation. After the five day incubation, sensitized effectors were harvested by gentle decantation, and washed one time in 20 mL of room temperature HBSS at 400 x g, 10 min, 23 °C. After enumeration and viability assessment, cells were resuspended to 8.0×10^6 cells/mL.

Cytotoxic T-Cell Reaction. Sensitized effector and target cells were combined in a 96-well round-bottom microtiter plate (Fisher) in duplicate E:T ratios of 1:1, 2:1, 4:1, and 8:1, with target cell numbers consistently plated to 5.0×10^4 cells per well. To ensure chromium release was actually due to cytotoxic T-cell activity, minimum and maximum chromium release wells were plated in duplicate by adding either 100 mL media or 2% NP-40 detergent, respectively, to 5.0×10^4 target cells per well. Plates were centrifuged at 350 x g, 2 min, 23 °C to insure close cell-to-cell contact, and incubated at 28 °C, 5% CO₂, 95% humidity for 4 hours to allow sensitized effectors to recognize and lyse targets.

Gamma Release Determination. After incubation, plates were gently agitated to suspend cells, then centrifuged at 500 x g, 5 min, 23 °C to pellet cells. The supernatant containing the released chromium was collected using Skatron® filters and tubes, then gamma radioactivity was assessed via counts per minute (cpm) in a Beckman Gamma 5500 counter (Beckman Instruments, Inc., Schaumburg, IL). Samples were read twice, and the average cpm per sample

was calculated. The percent of specific gamma release was calculated using the equation (Hudson and Hay, 1989):

$$\% \text{ Specific Release} = 100 \times \frac{\text{CPM}_{\text{exp}} - \text{CPM}_{\text{min}}}{\text{CPM}_{\text{max}} - \text{CPM}_{\text{min}}}$$

Statistical Analysis. Experiments were designed under normal analysis of variance parameters (Sokal and Rohlf, 1995) and were analyzed using a SAS computer analysis package designed especially for this application, employing cross-wise comparisons using Tukey's and Student's-t analyses, and a significance level of $P < 0.05$.

Results

All fish which received vehicle only presented as clinically normal throughout the dosing regimens. Gross examinations of the pronephros tissue of the positive controls presented a typical, dark-reddish color.

Dose range-finding studies were conducted in fish to determine levels producing overt toxicity (increased mortality; abnormal swimming behavior; temporary loss of equilibrium). No more than 50% of an overtly toxic level of chemical exposure was used in experiments monitoring humoral immune function. Transient limited toxicity in fish (hyperpigmentation of skin

covering scales and fins, slight lethargy, and temporary reduction of feed intake) were observed after exposure to some of the selected chemical agents.

The pronephric tissue of the vehicle-exposed fish and fish dosed with acetonitrile, formaldehyde, oxymethalone, and TBHQ appeared dark reddish in color (clinically normal). The pronephric tissue of the fish dosed with azathioprine, B[a]P, DMBA, TCDD, DMN, DES, CdCl, T₂ toxin, lindane, and toluene typically was more friable and appeared dark reddish-brown in color.

During the cytolytic portion of the assay where the sensitized effectors were mixed with the chromium-labeled targets, preliminary assay development proved an effector-target ratio of 1:1 to be most effective in the tilapian assay.

CTL Activity. In fish dosed with azathioprine, B[a]P, DMBA, DMN, CdCl, T₂-toxin, TCDD, toluene, and lindane, cpm numbers were decreased significantly as compared to the vehicle dosed fish (Figures 1-9). Fish exposed to acetonitrile, DES, oxymethalone, formaldehyde and TBHQ showed no significant difference when compared to vehicle-dosed controls (Figures 10-14).

P-values are given as follows:

Σ Azathioprine	P=0.0050
Σ B[a]P	P=0.0050
Σ DMBA	P=0.0020
Σ DMN	P=0.0450

Σ CdCl ₂	P=0.0190
Σ T ₂ -toxin	P=0.0020
Σ TCDD	P=0.0009
Σ Lindane	P=0.0051
Σ Toluene	P=0.0498
Σ Acetonitrile	P=0.9000
Σ DES	P=0.3468
Σ Oxymethalone	P=0.3780
Σ Formaldehyde	P=0.2290
Σ TBHQ	P=0.4680

Discussion

We recently found that the humoral immune response in fish (i.e., the ability to produce specific antibody following challenge with antigen) was depressed in an manner similar to that reported in laboratory rodents following challenge with a diverse group of immunotoxic compounds (Smith et al., submitted). Specifically, fish were dosed with nine compounds defined as rodent immunotoxicants and five compounds presently considered to be non-immunotoxic (Luster et al., 1988). For 13 of these 14 compounds (eight of the nine immunotoxicants, with the exception of lindane, and five of the five non-immunotoxicants) fish responded in the same manner as rodents, i.e., the

immunotoxicants depressed antibody production, while the non-immunotoxicants gave results similar to controls.

Therefore, we continued our study by evaluating the CTL assay in fish, which assesses the T-cell response to dissimilar MHC antigens, and is the *in vitro* counterpart to the graft versus host (GvH) reaction (Colegan et al., 1991). This assay carries a strong individual predictive value for immunosuppression in rodents, and as well, has a very high pairwise predictive value when combined with the PFC assay (100%) (Luster et al., 1992). Further, the CTL assay has previously been developed in catfish (Faisal et al., 1989; Stuge et al., 1997, and Yoshida et al., 1995), and thus was available for use with minor modifications in tilapia.

The cell-mediated immune response requires a complex interaction between antigen presenting cells, T-helper cells, cytotoxic T-cells, and various interleukin mediators. Immunotoxic chemicals modulate the functions of these components by altering antigen processing and/or presentation, cell proliferation and/or differentiation, or synthesis and/or release of cytokines (Luster et al., 1992). The CTL assay employs purified lymphocytes from the tested animal, mixed with lymphoblasts, tissue culture cells, or tumor cells which possess dissimilar MHC proteins, i.e., cells from an allogeneic individual of the same species (target cells). Previous sensitization of the tested animal with the target cells *in vivo* is not necessary for an adequate response, because as many as 10% of the T-cell population will respond without prior sensitization

to the presence of foreign MHC molecules, eliciting a large cytolytic response directed toward the target cells (Colegan et al., 1991).

Our results showed that azathioprine, B[a]P, DMBA, CdCl₂, lindane, T₂ toxin, and TCDD strongly depressed CTL activity in fish, similar to the effects of these chemicals in rodents (Luster et al., 1992). DMN and toluene also produced significant depression in CTL cytolytic capacity, again similar to the effect of these agents in rodents (Luster et al., 1992; Cheremisinoff et al., 1994; Klaassen, 1996; and Smialowicz and Holsapple, 1996). One chemical, DES, which is known to inhibit CTL activity in rodents (Smialowicz and Holsapple, 1996), failed to depress CTL activity in fish. This discrepancy may have been caused by differences in dosing parameters employed. Alternately, fish immunity may respond differently to DES than rodents. All four chemicals considered to be non-immunotoxic (acetonitrile, formaldehyde, oxymethalone, and TBHQ) had no effect on CTL activity, again similar to the rodents model.

In summary, previous results in fish with the PFC assay and the present results with the CTL assay raise questions regarding the efficacy of using standard rodent immunotoxicity assays for detecting immunosuppression in fish. The PFC assay in fish indicated that all rodent humoral immunotoxicants except one (hexachlorocyclohexane, or lindane) also produced humoral immunosuppression in fish. Although hexachlorocyclohexane depresses antibody responses in rodents, it is interesting to note that this compound did not depress antibody production in carp (Cossarini-Dunier et al., 1987). Thus it

may be that this chemical agent, for undetermined reasons, does not depress antibody production in fish.

The CTL assay in fish gave results overall quite similar to the PFC results, with nine of ten chemical agents previously demonstrated to depress this immune cell activity in mice also inhibiting the fish CTL response (diethylstilbesterol being the exception). All four of the compounds with negative CTL effects in rodents were also negative in fish. Thus, 13 of 14 chemical agents tested gave similar results in fish as reported in rodents, indicating a comparable pattern of T-cell mediated immunosuppression in chemical-exposed tilapia to that seen in laboratory rodents.

Interestingly, the two chemicals that did not produce comparable results to the rodent model with the tilapian PFC and CTL assays are both estrogenic compounds. It could therefore be hypothesized that tilapian estrogen receptors may carry less affinity for these compounds than do mammalian receptors, however, lindane did produce strong cell-mediated immunosuppressive results with the CTL assay in tilapia, as did TCDD, which is also known to be estrogenic. Further, it is not known if binding to the estrogen receptors is a major mechanism of action with these chemicals. Increasing the dosage over a greater period of time with DES and lindane in tilapia to determine if immunosuppressive results will ensue will give more insight into the question of whether fish are affected immunologically by these chemicals.

The present data developed with the PFC and CTL assay in tilapia indicate that tilapia often respond to immunotoxicant exposure in a manner similar to rodents, and thus, that fish may have the potential for further development as an alternate species to mammals for use in preliminary chemical immunotoxicant detection and immunotoxicity assessment.

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TABLES AND FIGURES

Table 1: Dosage dilutions and amounts of chemicals and vehicles used for the PFC assay in tilapia.

Figure 1: Effects of azathioprine on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 10.0 mg/kg AZA on experimental day 7. Vehicle exposed fish received saline on experimental day 7. Reduced counts per minute were observed in AZA treated fish. * indicates significantly different from the vehicle dosed group, $P=0.0050$.

Figure 2: Effects of B[a]P on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 5.0 mg/kg B[a]P on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. Reduced counts per minute were observed in B[a]P treated fish. * indicates significantly different from the vehicle dosed group, $P=0.0050$.

Figure 3: Effects of DMBA on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using

the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 5.0 mg/kg DMBA on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. Reduced counts per minute were observed in DMBA treated fish. * indicates significantly different from the vehicle dosed group, P=0.0020.

Figure 4: Effects of DMN on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 1.0 mg/kg DMN on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. Reduced counts per minute were observed in DMN treated fish. * indicates significantly different from the vehicle dosed group, P=0.0450.

Figure 5: Effects of CdCl on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 1.0 mg/kg CdCl on experimental day 7. Vehicle exposed fish received distilled water on experimental day 7. Reduced counts per minute were observed in CdCl treated fish. * indicates significantly different from the vehicle dosed group, P=0.0190.

Figure 6: Effects of T₂ on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using

the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 0.1 mg/kg T₂ on experimental day 7. Vehicle exposed fish received corn oil plus EtOH on experimental day 7. Reduced counts per minute were observed in T₂ treated fish. * indicates significantly different from the vehicle dosed group, P=0.0020.

Figure 7: Effects of TCDD on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 1.0 mg/kg TCDD on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. Reduced counts per minute were observed in TCDD treated fish. * indicates significantly different from the vehicle dosed group, P=0.0009.

Figure 8: Effects of lindane on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 20.0 mg/kg CdCl on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. Reduced counts per minute were observed in lindane treated fish. * indicates significantly different from the vehicle dosed group, P=0.0051.

Figure 9: Effects of toluene on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 0.5 mg/kg toluene on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. Reduced counts per minute were observed in toluene treated fish. * indicates significantly different from the vehicle dosed group, $P=0.0498$.

Figure 10: Effects of acetonitrile on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 10.0 mg/kg acetonitrile on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. No reduction in counts per minute was observed in acetonitrile treated fish, $P=0.9000$.

Figure 11: Effects of DES on the tilapian cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 6.0 mg/kg DES on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. No reduction in counts per minute was observed in DES treated fish, $P=0.3468$.

Figure 12: Effects of oxymethalone on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 1.0 mg/kg oxymethalone on experimental day 7. Vehicle exposed fish received corn oil on experimental day 7. No reduction in counts per minute was observed in oxymethalone treated fish, $P=0.3780$.

Figure 13: Effects of formaldehyde on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 100 ppm formaldehyde in 20 gal of water for 30 minutes on experimental day 7. Vehicle exposed fish were placed in 20 gal of water without formaldehyde for 30 minutes. No reduction of counts per minute was observed in formaldehyde treated fish, $P=0.2290$.

Figure 14: Effects of TBHQ on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay. Chemical-exposed fish received 10.0 mg/kg TBHQ on experimental day 7. Vehicle exposed fish received distilled water on experimental day 7. No reduction of plaque formation was observed in THBQ treated fish, $P=0.4680$.

TABLE 1

**Dosage dilutions and amounts of chemicals and vehicles
used for the PFC assay in tilapia**

Chemical	Dilution	Vehicle	Average Dosage Amount
Acetonitrile	10.0 mg/kg	dWater	0.45 mL
AZA	10.0 mg/kg	Saline	0.70 mL
B[a]P	5.0 mg/kg	Corn Oil	0.15 mL
CdCl	1.0 mg/kg	dWater	0.45 mL
DES	6.0 mg/kg	Corn Oil	0.25 mL
DMBA	5.0 mg/kg	Corn Oil	0.30 mL
DMN	1.0 mg/kg	Corn Oil	0.50 mL
Formaldehyde	100 ppm	Water	8 mL/20 gal
Lindane	20.0 mg/kg	Corn Oil	0.55 mL
Oxymethalone	1.0 mg/kg	Corn Oil	0.50 mL
T ₂	0.1 mg/kg	Corn Oil + ETOH	0.15 mL
TBHQ	10.0 mg/kg	dWater	0.45 mL
TCDD	1.0 mg/kg	Corn Oil	0.35 mL
Toluene	0.5 mg/kg	Corn Oil	0.35 mL

FIGURE 1

Effects of azathioprine on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

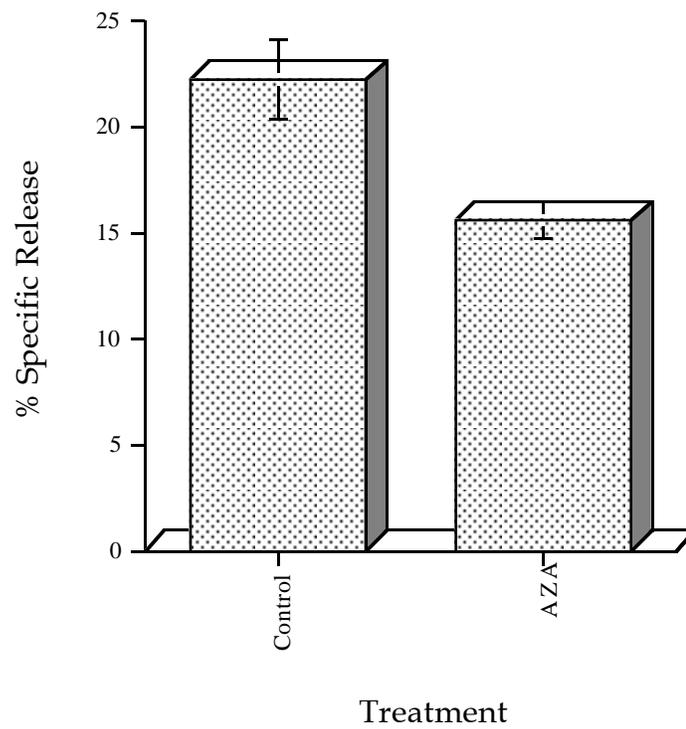


FIGURE 2

Effects of B[a]P on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

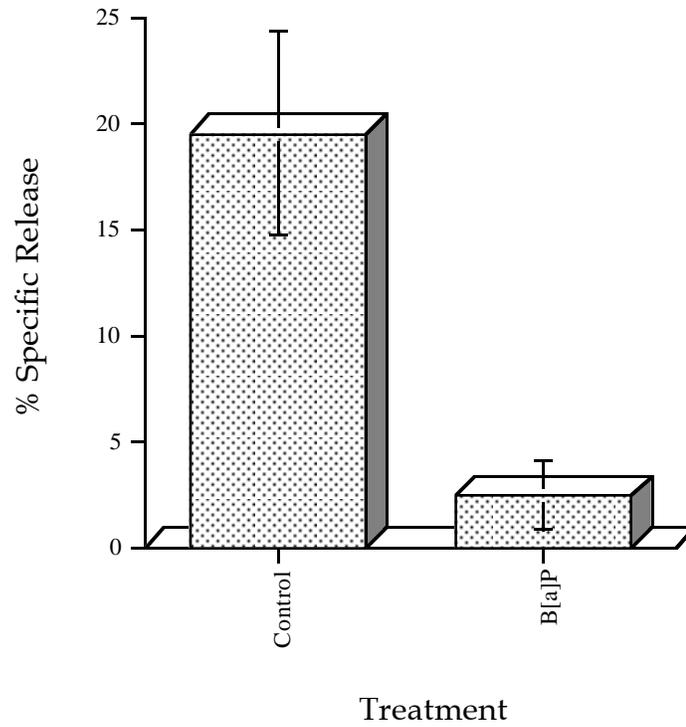


FIGURE 3

Effects of DMBA on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

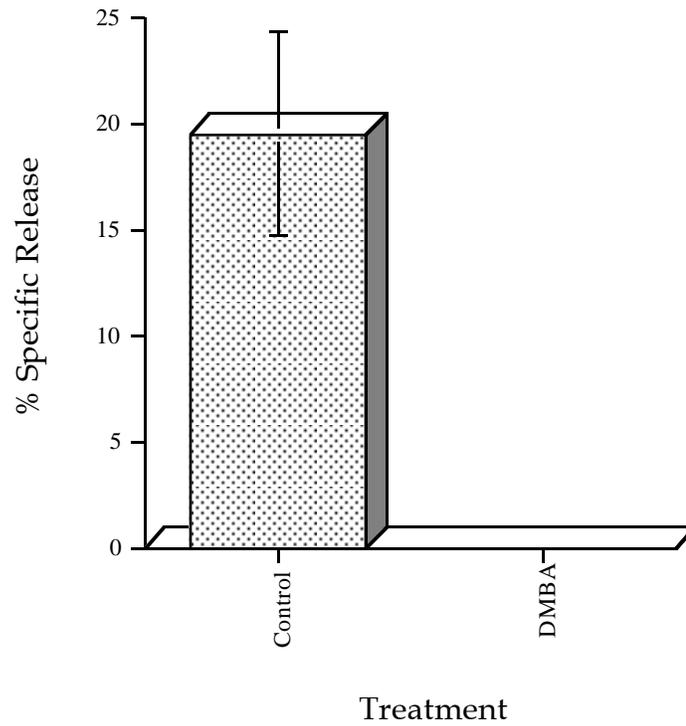


FIGURE 4

Effects of DMN on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

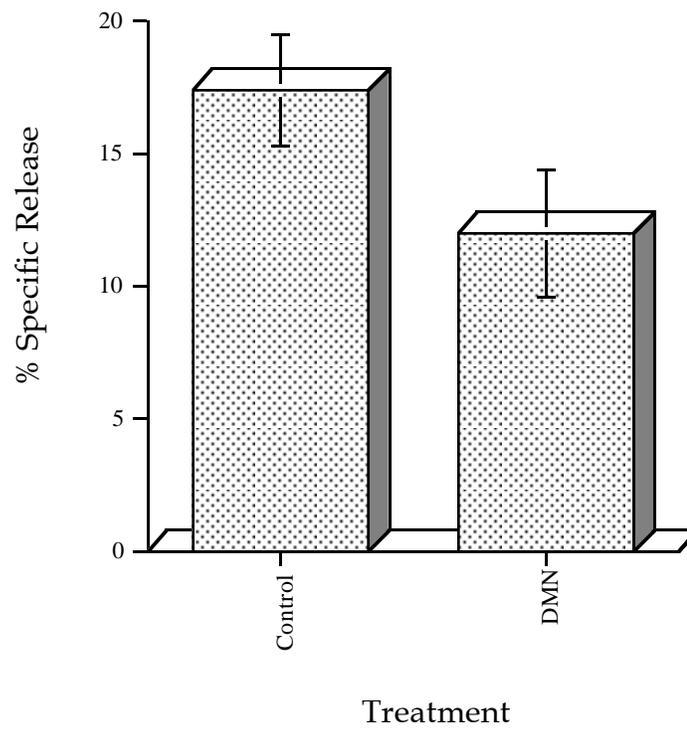


FIGURE 5

Effects of CdCl on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

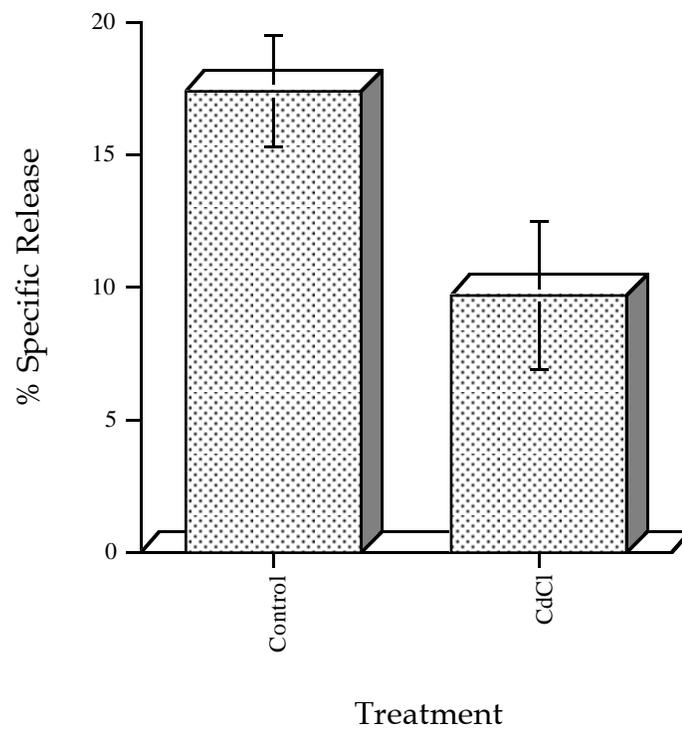


FIGURE 6

Effects of T₂-toxin on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

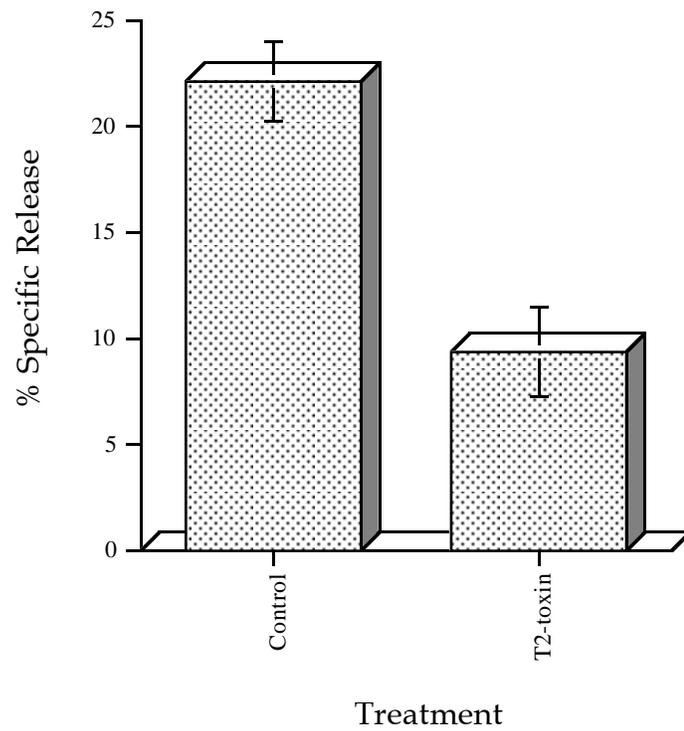


FIGURE 7

Effects of TCDD on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

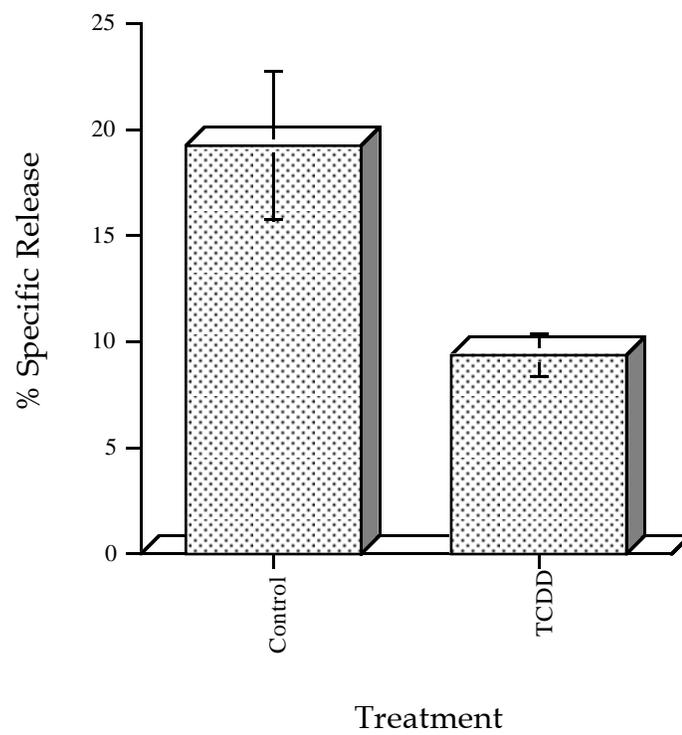


FIGURE 8

Effects of lindane on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

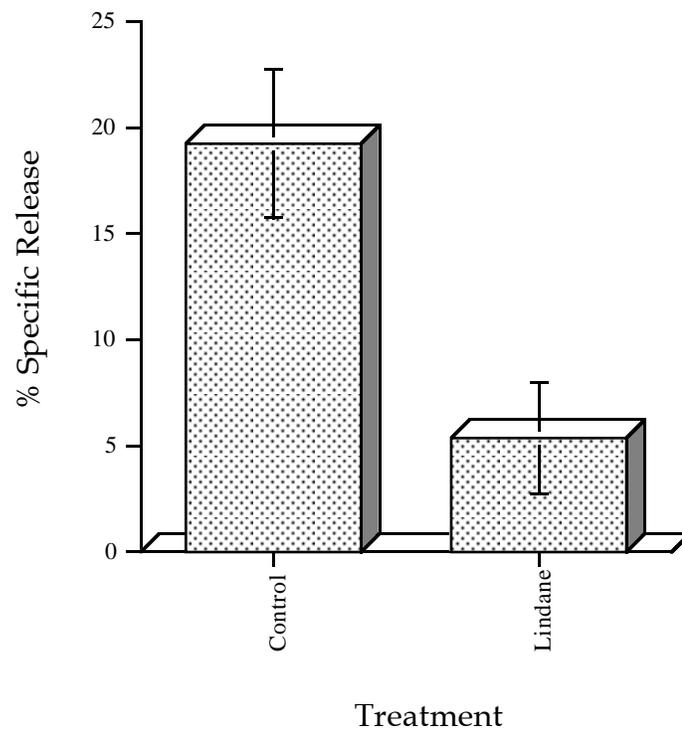


FIGURE 9

Effects of toluene on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

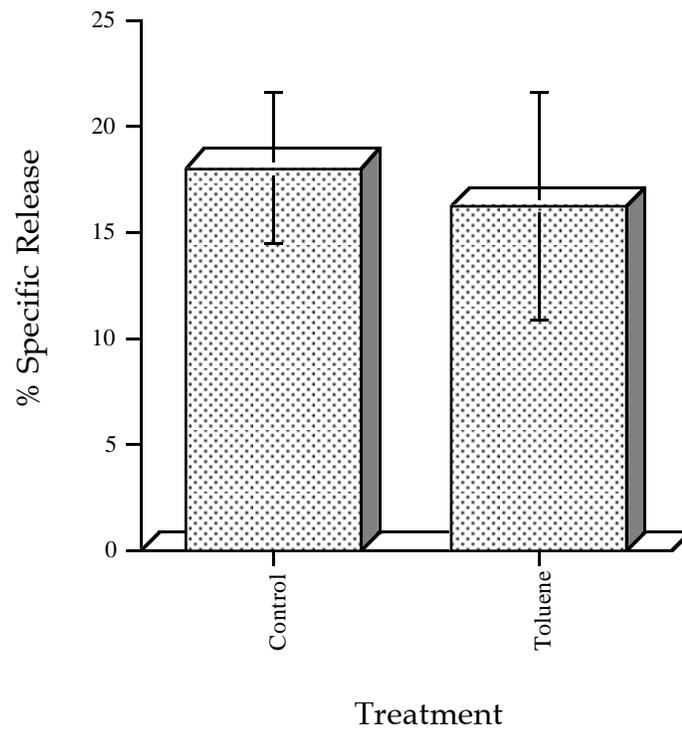


FIGURE 10

Effects of acetonitrile on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

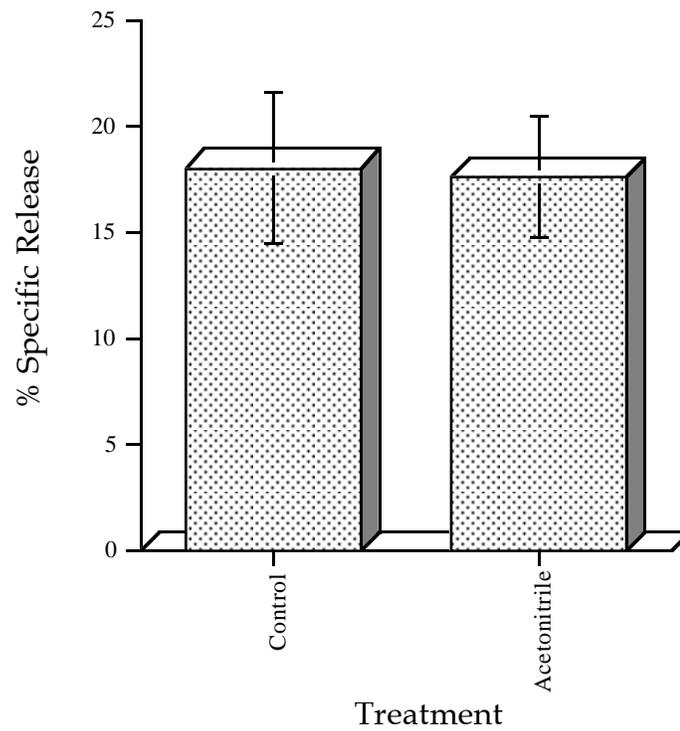


FIGURE 11

Effects of DES on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

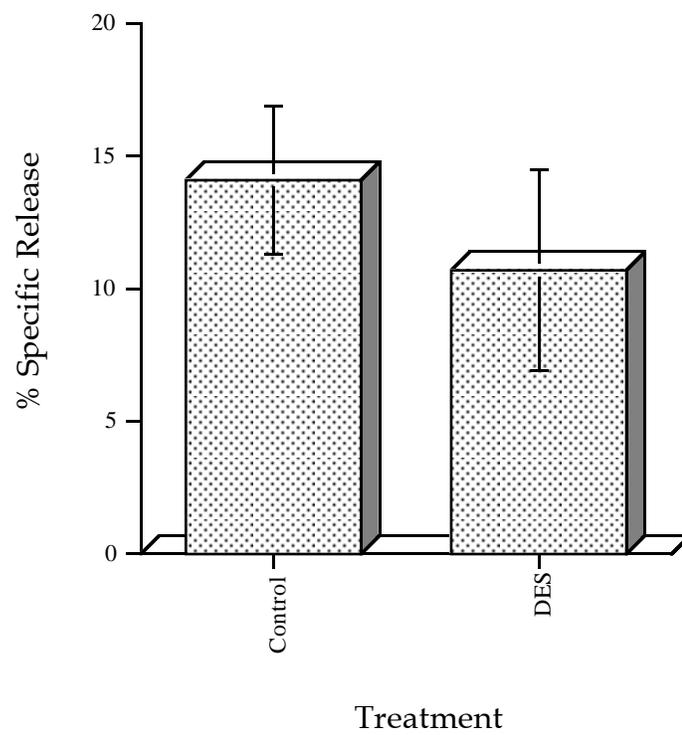


FIGURE 12

Effects of oxymethalone on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

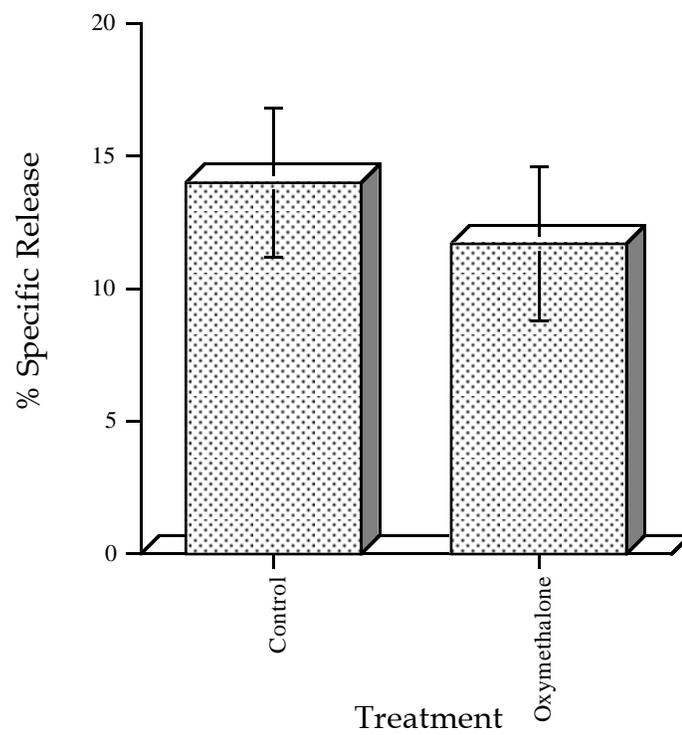


FIGURE 13

Effects of formaldehyde on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay

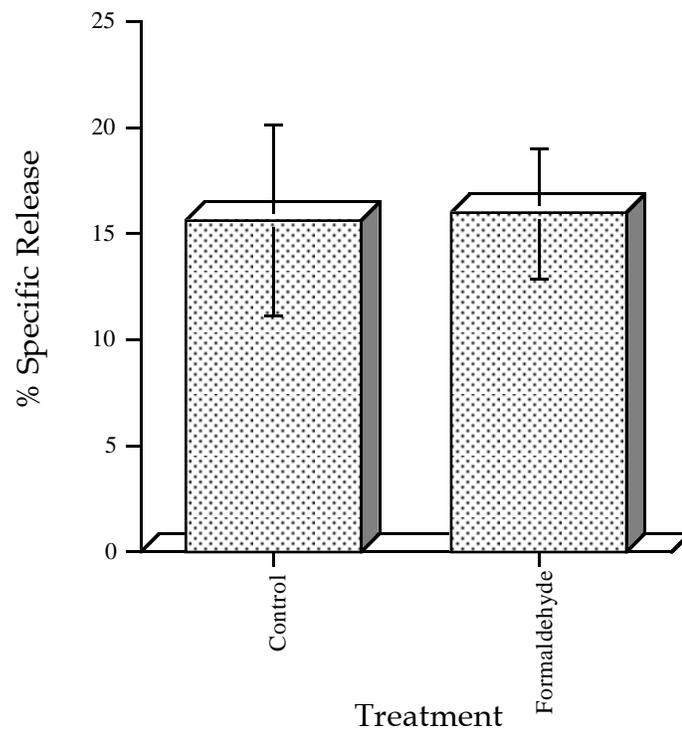
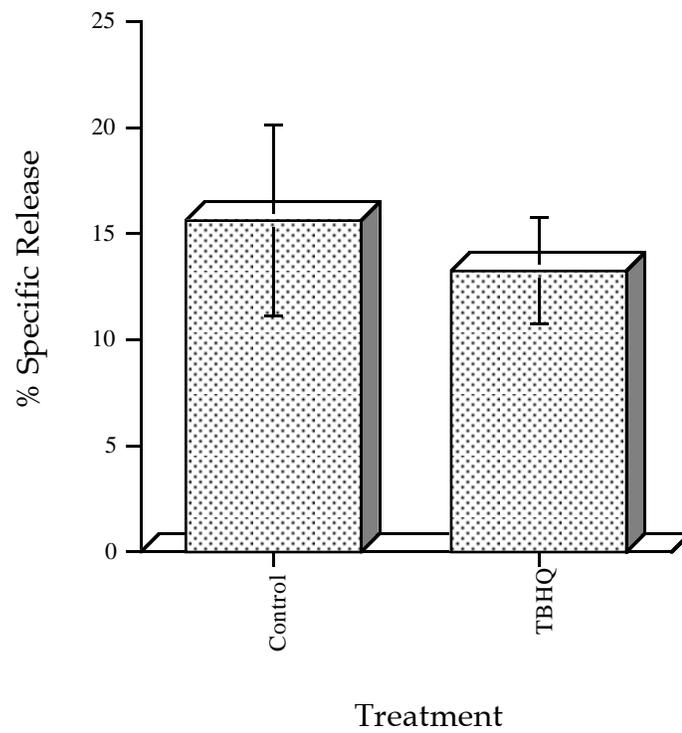


FIGURE 14

Effects of TBHQ on the tilapia cell-mediated response to allogeneic lymphocytes as indicated by the % specific release of gamma radiation using the cytotoxic T-lymphocyte assay



SUMMARY

The hemolytic plaque forming cell (PFC) assay (a measure of ability to produce specific antibody following challenge with antigen) and the cytotoxic T lymphocyte (CTL) assay (a measure of the ability of cytotoxic T cells to target and lyse cells expressing foreign antigen) represent two of the most powerful individual predictors of immunosuppression in toxicant-exposed laboratory rodents. Furthermore, these two assays have been found to have a pairwise predictive value for detecting immunosuppression in B6C3F1 mice of 1.0 (i.e., in all cases where a chemical agent produces inhibition of both assays, challenge assays have demonstrated clinically significant immunosuppression in the mice). However, the ability of these assays to detect chemical-induced immuno- suppression in non-rodent species remains unknown. Therefore, the PFC and CTL assays were modified for use in a widely cultured and economically important tilapian fish species, *Oreochromis niloticus*. The focus of the present research was two-fold: 1) To begin to characterize in a controlled laboratory setting the relative effect of a chemically-diverse group of known rodent immunotoxicants on fish immune function, and 2) To evaluate the use of traditional rodent immunotoxicity risk assessment methodologies in fish as possible sensitive indicators of immune impairment.

The PFC and CTL assays were developed for use in tilapia, and then tested in the fish model using fourteen chemical agents selected from the National

Toxicology Program (NTP) list of rodent immunotoxicants and non-immunotoxicants. Chemically-diverse immunotoxicants were purposefully selected, and included polycyclic aromatic hydrocarbons, a halogenated aromatic hydrocarbon, a potent estrogen, a heavy metal, a chlorinated insecticide, a fungal mycotoxin, an alkylating agent, and a human therapeutic immunosuppressant. Five agents considered by the NTP to be non-immunotoxic to rodents were included as negative controls.

Eight of the nine test chemicals with previously demonstrated humoral immunotoxic activity in mice caused a depressed PFC response in tilapia (hexachlorocyclohexane being the exception). All five agents which do not reduce antibody production in mice produced similar negative results in tilapia. Thus, 13 of 14 agents tested gave similar results in fish as reported in rodents, indicating the humoral immune response of chemical-exposed tilapia compares well with rodents.

The CTL assay in fish gave results overall quite similar to the PFC results, with nine of ten chemical agents previously demonstrated to depress this immune cell activity in mice also inhibiting the fish CTL response (diethylstilbesterol being the exception). All four of the compounds with negative CTL effects in rodents were also negative in fish. Thus, 13 of 14 chemical agents tested gave similar results in fish as reported in rodents, indicating a comparable pattern of T-cell mediated immunosuppression in chemical-exposed tilapia to that seen in laboratory rodents.

Challenge assays were not included as endpoints in the present study design, thus it is unknown if the two "false negative" chemicals (hexachlorocyclohexane and diethylstilbesterol) are true immunotoxicants in fish (i.e., if non-overtly toxic levels of these agents will produce immunosuppression in fish, as is the case in mammals). Given that false negative results are generally of greater concern in a preliminary risk assessment model than are false positives, the idea that fish might be used as alternates to rodents to screen for potential mammalian immunotoxic chemicals must be considered with some caution. Hexachlorocyclohexane (clearly an immunosuppressive chemical in rodents) produced immunosuppressive results with one of the two fish immune tests considered, the CTL assay. Thus, if both the CTL and PFC assays are conducted, hexachlorocyclohexane would have been correctly identified as a potential rodent immunotoxicant using a fish model. However, the chemical diethylstilbesterol is well characterized as a cell-mediated immunotoxicant in both rodents and humans, but did not depress the CTL assay in tilapia. With refinement of the fish model, it is possible that an even closer parallel to rodents could be obtained (i.e., use of different dosing regimes, chemical exposure levels, etc.) might demonstrate humoral immunotoxicity with hexachlorocyclohexane and a depressed CTL response with diethylstilbesterol. Alternately, fish immunity may for undetermined reasons respond differently than rodents to hexachlorocyclohexane and diethylstilbesterol. If continued

research demonstrates that such is the case, the implication is that chemical immunotoxicity risk assessment using fish may fail to detect certain mammalian immunotoxicants.

VITAE

Dorinda Ann Smith was born on December 7, 1962 in San Diego, California, and was raised in the suburb of Lakeside. After graduating from Crawford High School in 1979, Ms. Smith founded a successful singing business while devoting her efforts towards her home and family.

In 1991 her life-long goal of entering college to pursue a career as a research scientist became a reality, and she received her Bachelor of Science degree in Biology with Emphasis in Immunology and Microbiology and a minor in Chemistry from Virginia Polytechnic Institute and State University in May of 1996. Her fascination with animal and human immune function led to the pursuit of a Master of Science degree in Veterinary Medical Sciences with Dr. Steven D. Holladay at the Virginia-Maryland Regional College of Veterinary Medicine, studying fish and rodent immunotoxicology. Her education is highlighted by receiving the first place award at the 14th Annual Virginia Tech Research Symposium for the development of a modified PFC assay in the tilapia fish species, and maintaining Cum Laude status.

Dorinda will be continuing her education by pursuing her Ph.D. in the laboratory of Dr. Dori Germolec at the National Institute of Environmental Health Sciences (NIEHS) in Research Triangle Park, North Carolina in August, 1998. Her focus of study will be linking environmental contaminants/endocrine disruptors to increased incidences of autoimmune disease.