Use of the Immune System to Investigate the Toxicity Induced by Environmental Pollutants in Fish, Amphibian, and Mammalian Species

Lisa Michelle Hudson Pélanne

Master's Thesis submitted to the Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Klaus D. Elgert, Chair
Alan G. Heath
Steven D. Holladay

10 April 2002
Blacksburg, Virginia

Acknowledgements

I would like to acknowledge my graduate committee chairperson, Dr. Klaus Elgert, and my committee members, Dr. Alan Heath and Dr. Steven Holladay for their support and assistance in the completion of the Master of Science degree. I also acknowledge my former advisor, Dr. Prakash Nagarkatti, and my former co-advisor, Dr. Mitzi Nagarkatti, for the opportunity to pursue research in their lab. I thank my former committee member Dr. Barbara Davis for her support. Additionally, I acknowledge Dr. Joe Cowles for his support.

I thank Dr. Don Orth, Van Stancil, Matt Chan, Brett Albanese, and all of the fisheries students who contributed greatly to the field research. The histological studies could not have been done without the help and expertise of Dr. Thomas Caceci, Dr. Bob Duncan, and Dr. John Robertson. I wish to thank Dr. Don Linzey for providing the frog and toad samples from Bermuda, as well as including me on his research project.

I also thank Ms. Joan Kalnitsky for her assistance with the flow cytometry, and Chris Cohen and Jenny Sutphin for the care of the animal facilities. I thank Dr. Donald Jensen and Ed Boone of the Statistical Consulting Center of Virginia Tech for their assistance in statistical analysis. Thanks are also due to the support staff of the biology department of Virginia Tech, especially Ms. Sue Rasmussen.

Thanks to all the graduate students and postdoctoral students at Virginia Tech who assisted me with my research projects. Special thanks are due to my husband, Philippe Pélanne, as well as family and friends for their support.

I would like to thank the Environmental Protection Agency, National Science Foundation, Waste Policy Institute, and Sigma Xi Grants-in-aid of Research for funding this research.

Use of the Immune System to Investigate the Toxicity Induced by Environmental Pollutants in Fish, Amphibian, and Mammalian Species

By

Lisa Michelle Hudson Pélanne

Department of Biology

Abstract

In recent years, there has been growing concern about the effect of environmental pollutants on the immune system. In the current study, we investigated the toxicity induced by certain environmental pollutants on the immune systems of fish, amphibians, and mice. Fish in the laboratory were tested for susceptibility to immunosuppression by treatment with 1,3-Bis (chloroethyl)-1-nitrosourea (BCNU). Immunotoxicity of the tilapian immune system was detectable using mitogen-induced proliferation assay and cell-mediated toxicity assay. Fish from various streams of the Roanoke River were tested for immunotoxicity and parasitic infection. Fish from the more polluted North Fork of the Roanoke River exhibited a stronger mitogenic response when compared to fish from the South Fork of the Roanoke River. The effect of perinatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), commonly referred to as dioxin, a highly toxic environmental pollutant, was tested in C57BL/6 mice. TCDD was administered on gestational day 14 and pup thymocytes were studied for apoptosis on postnatal days 2, 4, 7, 14, and 21. Perinatal exposure to TCDD decreased thymic cellularity and induced apoptosis in the thymocytes of the pups. Amphibians from polluted areas of Bermuda were similarly tested for immunotoxicity and compared with amphibians from less polluted areas. The lymphocyte responsiveness of toads from the more polluted Bermuda Biological Station of

Research (BBSR) to mitogens such as lipopolysaccarhide (LPS) was significantly less than in toads from less polluted areas of Bermuda. Histological studies revealed differences in the liver and spleen tissues of the two groups. Melanomacrophage centers were prevalent in the livers of amphibians from the more polluted BBSR when compared to the less polluted Zoo site.

These data taken together encompass a broad study on the effect of environmental pollutants across species. In each study, immunotoxicity is the end result of contact with contamination, whether occurring in the environment or induced in the laboratory. These data suggest that the immune system may serve as a biomarker for pollutants present in the environment.

Table of Contents

List of Illustrations

List of Abbreviations

Ah, aryl hydrocarbon

APC, antigen presenting cell

BCNU, 1,3-Bis (chloroethyl)-1-nitrosourea

CD, cluster of differentiation

CMI, cell-mediated immunity

CTL, cytotoxic T lymphocyte

DN, double negative

DNA, deoxyribonucleic acid

DP, double positive

DRE, dioxin responsive element

FcR, Fc receptor

FITC, fluorescein isothiocyanate

H&E, hemotoxylin and eosin

HAH, halogenated aromatic hydrocarbons

HS, heat shock

i.p., intraperitoneally

IFN, interferon

Ig, immunoglobulin

IL, interleukin

LN, lymph node

mAb, monoclonal antibody

MHC, major histocompatibility complex

MFI, mean fluorescent intensity

NK cell, natural killer cell

PALS, periarteriolar lymphoid sheath

PBS, phosphate buffered saline

PE, phycoerythrin

RPMI, Roswell Park Memorial Institute culture medium

SD, standard deviation

SDS, sodium dodecyl sulfate

SEM, standard error of the mean

TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TCR, T cell receptor

TdT, terminal deoxynucleotidyl transferase

TGF, transforming growth factor

T_h, T helper cell

TNF, tumor necrosis factor

TUNEL, TdT-mediated nick end labeling

URRW, Upper Roanoke River Watershed

Chapter 1: Introduction

The Immune System

Immunology is the study of the complex cellular and chemical reactions that occur in a host animal in response to contact with a foreign entity, such as a pathogen. An immune response involves recognition of the pathogen by the host as foreign and the subsequent immune response to eliminate it (Roitt, 1998).

There are two types of immune response, innate and adaptive. The innate response, which does not retain memory upon repeated exposure to the pathogen, is not specific to the pathogen. The adaptive immune response is specific to the pathogen and has memory upon repeated exposure.

The vertebrate immune system is composed of two primary immune organs, the bone marrow and the thymus, which provide appropriate environments for lymphocyte maturation. Secondary immune organs such as the spleen, lymph nodes, and mucosa-associated lymphoid tissue (MALT) trap antigen and provide appropriate microenvironments for lymphocyte-antigen interaction. Birds have an additional immune organ known as the Bursa of Fabricius (Roitt, 1998). Fish have no bone marrow; so production of lymphocytes occurs in the anterior kidney (pronephros), gut-associated tissue, and spleen (Heath, 1995). It is also noteworthy that fish do not have lymph nodes.

The thymus is a highly lobulated, bilobed organ in which T-cell maturation occurs. Each lobe consists of an outer cortex and inner medulla. The cortex is packed with lymphocytes while the medulla has fewer lymphocytes and contains primarily epithelial tissue. The prime functions of the thymus include: development of immunocompetent T lymphocytes from bone marrow

precursors, differentiation of T cell subsets, proliferation of clones of mature naive T cells, development of immunological self-tolerance, and secretion of hormones such as thymulin and thymopoietin and other factors which regulate T-cell maturation, proliferation, and function (Burkitt, 1993). The thymus undergoes a process of involution as an animal matures due to infiltration of adipose tissue and depletion of lymphocytes (Burkitt, 1993).

The lymph nodes are small organs situated in the course of the regional lymphatic vessels. The lymph nodes are structurally divided into the cortex, paracortex, and medulla. The cortex is highly cellular and is densely packed with lymphoid follicles, some with germinal centers. The paracortex, or deep cortex, is devoid of lymphoid follicles. The inner medulla is less cellular and its medullary cords extend into the cortex. The functions of the lymph nodes include: nonspecific filtration of particulate matter and pathogens from lymph by the phagocytic activity of macrophages, interaction of circulating lymphocytes with antigen-presenting cells in the node, and aggregation, activation and proliferation of B and T lymphocytes after appropriate antigenic stimulation (Burkitt, 1993).

The spleen has two primary functions: production of immune responses against blood-born antigens, and removal of particulate matter and aged or defective erythrocytes from the circulation. The spleen consists of areas of red and white pulp. The red pulp consists of cords and sinuses populated with macrophages. The white pulp is found surrounding the arterioles and is referred to as the periarteriolar lymphoid sheath (PALS). In humans, T lymphocyte masses are located around arterioles and B lymphocyte follicles lie adjacent to the arterioles in an irregular fashion (Burkitt, 1993).

The cells of the immune system evolve from pluripotent hematopoietic stem cells found in the bone marrow. Known as leukocytes, or “white blood cells,” the immune cells are

classified as either granulocytes or agranulocytes. The granulocytes include the basophils, mast cells, eosinophils, and neutrophils, and the agranulocytes include the monocytes, Natural Killer (NK) cells, and lymphocytes. The lymphocytes are further divided into the B lymphocytes and T lymphocytes (Roitt, 1998).

Neutrophils constitute the majority of circulating lymphocytes. Being highly motile and phagocytic, they are the first to arrive at a site of inflammation. Eosinophils constitute 1-6% of circulating lymphocytes. They have a phagocytic function, are involved in parasitic infection, as well as play a role in ameliorating some aspects of hypersensitivity. Basophils are the least common circulating leukocyte and play a role in allergic reaction. Mast cells are found in the tissues and play a similar role in allergic reaction (Burkitt, 1993).

Monocytes are the largest of the circulating leukocytes. They are highly motile and phagocytic. Monocytes are the precursors to tissue-dwelling macrophages. Dendritic cells are antigen-presenting cells that express high levels of class II major histocompatibility complex (MHC)-encoded molecules. They are found in both lymphoid and non-lymphoid tissues. Natural Killer cells are involved in nonspecific killing of tumor cells and virally infected cells. NK cells play an important role in immune function by killing cells that lack MHC molecules (Burkitt, 1993).

There are two main divisions of the adaptive immune system, humoral immunity and cell-mediated immunity. Humoral immunity is mediated by B lymphocytes, which differentiate into plasma cells and produce antibodies. B lymphocytes mature in the bone marrow and are responsible for producing antibodies, which recognize and bind directly to specific antigens. Antibodies are composed of two light and two heavy chains and have a highly polymorphic variable domain. There are five classes of antibodies, or immunoglobulins: IgG, IgM, IgD, IgA,

and IgE. The precise biological function of IgD, which is found in large quantities on the membrane of B cells, is unknown. IgM is the first antibody to respond to foreign antigen. IgG accounts for 70-75% of the immunoglobulin in human serum. It is the major antibody of the secondary immune response. IgG is also the only antibody that crosses the placenta during pregnancy to confer passive immunity to the newborn. IgA represents 15-20% of the immunoglobulin pool and is the predominant antibody found in secretions. IgE is scarce in serum and is associated with allergic responses and immunity to helminth parasites (Roitt, 1998).

Cell-mediated immunity involves T lymphocytes. In mammals, prothymocytes travel from the bone marrow to the thymus where they mature into T cells. In the thymus, they undergo a positive and negative selection process to protect against self-recognition. In positive selection, thymocytes bearing receptors capable of binding to self-MHC molecules with low affinity are selected for, resulting in MHC-restriction. In negative selection, thymocytes bearing high affinity receptors for self antigens presented by MHC molecules are eliminated. There are four subpopulations of T cells in the thymus: $CD4^+CD8^+$, also known as double positive (DP), and $CD4^-CD8^-$, also known as double negative (DN), $CD4^+CD8^-$, known as T helper (T_h) cells, and $CD4^-CD8^+$, or cytotoxic T cells (CTLs). T cells recognize foreign antigens with the T cell receptor (TCR) in conjunction with the major histocompatibilty complex (MHC)-encoded molecule. There are two main classes of MHC molecule, MHC class I, which is associated with all nucleated cells, and MHC class II, which is associated with specialized antigen-presenting cells. All T lymphocytes bear the CD3 molecule, which is associated with the T cell receptor, throughout their lifespan (Roitt, 1998).

Immunotoxicology

The field of immunotoxicology is the study of the effects of chemicals on the immune system. Chemicals have either a suppressive effect on immune function, which can lead to increased susceptibility to infections and cancer, or they enhance the immune response, which can lead to increased autoimmunity or hypersensitivity. Most environmental pollutants are known to alter immune system function even at doses that do not produce organ toxicity. A broad spectrum of chemicals can affect the immune system (Vos 1977, 1980).

Fish Studies

In recent years, there has been growing concern about the health status of aquatic species in relation to environmental pollution. Fish may serve as an important biomarker for pollution (Wester, et al., 1994). They represent the largest and most diverse group of vertebrates, and fish have the ability to adapt to a wide variety of environments (Powers, 1989). Previous studies of fish toxicology have targeted ecological parameters such as population dynamics, more recently; however, attention has focused on toxicological pathways and other parameters at the cellular or tissue level (Wester, et al., 1994). Toxic effects on the immune system (immunotoxicity) may serve as an excellent tool and as a biomarker to monitor pollution and water quality. Wester, et al. (1994) describes the general characteristics important for fish as biomarkers as:

> sensitivity, a biomarker should reflect changes in the underlying causes, and within the order of magnitude relevant for the field situation; relevant and

informative; specific, with a cause and effect relationship established; easy, reproducible, and validated; and preferably measured by non-invasive methods. Thus, fish immunotoxicology may offer new tools to assess environmental water quality.

The main hypothesis tested in this study was that the fish immune system could serve as a useful biomarker to predict environmental pollution. The hypothesis was tested both in the field and in the laboratory using appropriate controls. Fish were collected from various streams of the Roanoke River drainage and the splenocytes were screened for their ability to respond to mitogens. Splenocyte proliferation was measured by DNA synthesis using the uptake of ^{3}H-thymidine. Secondly, NK cell functions were studied by their ability to kill YAC-1 tumor cells. Fish were sampled from streams that are contaminated with pollutants due to urbanization and compared to those sampled from streams that are less contaminated. The immunotoxicity data can then be correlated with the land use data, which ideally correlates with the levels of contaminants such as pesticides.

The lab studies were performed by injecting known immunosuppressive agents such as dexamethasone and BCNU intraperitoneally into fish and testing their immune functions. These results were used as comparison for the immune functions of fish from the field studies. Together these studies provide new and useful information on how immunotoxicological assays can serve as important biomarkers in the assessment of environmental pollution. These studies are useful not only to address the health status of aquatic species but also to extrapolate the data to human health concerns.

Amphibian Studies

Additionally, the immunotoxicological aspects of the marked decrease in amphibian populations worldwide, which many scientists attribute to exposure of the amphibians to contaminants, were investigated. Toads and frogs from Bermuda have undergone serious decline in the past several years. Amphibians from more polluted areas of Bermuda exhibit developmental malformations and physical abnormalities when compared to those from lesser-polluted areas (Linzey, unpublished). Studies suggest that amphibians are increasingly susceptible to disease, and this susceptibility may be linked to immunosuppression (Carey, 1999). In 1997, development of the North American Amphibian Monitoring Program (NAAMP) was started to monitor amphibian populations and distribution. The North American Reporting Center for Reporting Amphibian Malformations (NARCAM) was also established in 1997 in response to the recent and abrupt decline in amphibian populations. In this study, the hypothesis tested was that pollutant-induced immunosuppression is occurring in amphibian populations contaminated with toxic chemicals. This could be a contributing factor in the population declines.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, commonly known as “dioxin” or TCDD, is the most biologically potent of the halogenated aromatic hydrocarbons (HAH), and is considered to be the most toxic chemical ever created (Holsapple, 1991). It is an environmental contaminant formed as a byproduct in the manufacture of products from chlorinated phenols or during

combustion of chlorinated materials. HAH are associated with herbicides, pulp and paper manufacturing, automobile exhaust from leaded gasoline, and combustion of municipal and industrial wastes (Holsapple, 1991). The immune system is highly sensitive to TCDD which has raised significant health concerns, especially considering that HAH persists in the environment (Goldstein, 1989). TCDD causes decreased immunocompetence, even at doses that do not produce organ toxicity, and induces thymic involution as well as suppress T and B lymphocyte functions (Luster, 1987, Rhile, 1997, Thomas, 1985, and Vos, 1980). The exact mechanism by which TCDD induces immunosuppression is unknown. TCDD and other HAH have profound immunotoxic effects on experimental animals, which resemble the immunosuppressive effects caused by corticosteroid hormones (Lundberg, 1991).

Similar to the steroid receptor model, TCDD is thought to bind to a cytosolic receptor that is a principal gene product of the aryl hydrocarbon (Ah) locus. Assisted by heat shock (HS) proteins it binds to specific dioxin regulatory element (DRE) in the nucleus where it then binds to DNA and induces pleiotrophic effects (Holsapple, 1991, Nebert, 1989, Whitlock, 1987, 1990). TCDD is a potent transcriptional regulator of several genes in a variety of tissues inducing the cytochrome p450IA1, transforming growth factor (TGF), nuclear estrogen receptor, interleukin-1B, and plasminogen activation inhibitor (Choi, 1991, Poland and Knutson, 1982, Sutter, 1991). Also, Ah-independent processes such as change in intracellular calcium or change in the activity of a kinase/phosphatase system (Al-Bayati, 1988, Peterson, 1979) act as possible mechanisms for TCDD-induced apoptosis.

TCDD induces thymic atrophy (Kamath, 1997), which hinders immune function due to decrease in T-cell production. TCDD induces the terminal differentiation of thymic epithelial cells, preventing thymocyte maturation in younger animals, and in adult animals produces a

profound suppression of the antibody response (Holsapple, 1991). There are several proposed mechanisms for the induction of TCDD-induced thymic atrophy including damage to extrathymic T cell precursors in bone marrow and fetal liver (Fine, et al., 1989, Fine, et al., 1990, Silverstone, 1994) and initiation of apoptosis (Kamath, 1997).

Apoptosis

Apoptosis is a type of cell death characterized by DNA fragmentation into oligonucleosomal-length fragments and widespread blebbing of the nuclear and plasma membranes, inducing cell death. It arises due to an elevation of cytosolic free calcium concentration leading to activation of a nuclear endonuclease (Fawthrop, 1991). TCDD may stimulate the synthesis of a protein that mediates calcium influx (McConkey, 1988). Apoptosis is easily detected in its early stages using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, commonly referred to as TdT-mediated nick end labeling or TUNEL technique (Boehringer Mannheim, Indianapolis, IN) (Kamath, 1997). The fluorescent dye labels the oligonucleosomal-length fragments and is detected using flow cytometry.

Dexamethasone and TCDD are dose-dependent inducers of apoptosis, but they activate cell death by different mechanisms, indicated by the variations in thymocyte proliferation and number after treatment (Lundberg, 1991). Fas and Fas ligand (FasL) are molecules which play an important role in apoptosis (Nagata, 1997). Rhile, et al. demonstrated that thymic atrophy is regulated by the Fas molecule inasmuch as Fas^- (C57BL/6 lpr/lpr) mice were less sensitive to TCDD-mediated immunosuppression when compared to wild type Fas^+ (C57BL/6) mice (Rhile, 1996). The mechanism by which Fas regulates TCDD-mediated immunotoxicity is not clear. It

is suspected that TCDD may activate cytokines involved in the upregulation of Fas or FasL (Rhile, 1996), thereby facilitating induction of apoptosis.

Kamath, et al. (1997) successfully demonstrated that TCDD induces apoptosis *in vivo*, and that TCDD-induced apoptosis can only be detected at early stages (8-12 hours) *in vivo*. Kamath, et al. speculated that *in vitro* induction of apoptosis by TCDD is not detected due to an indirect effect of TCDD. This effect is speculated to be the production of cytokines involved in apoptosis by cells other than the thymus. Fas or FasL may be involved, as FasL is upregulated following TCDD exposure (Kamath, 1999). TCDD increases levels of serum TNF, and may upregulate other cytokines (Kamath, 1997), involved in thymic apoptosis. TCDD-induced apoptosis may have significant impact on regulation of immunotoxicity mediated by dioxin and other halogenated aromatic hydrocarbons.

Specific Aims

The specific aims of this study, *Use of the Immune System to Investigate the Toxicity Induced by Environmental Pollutants in Fish, Amphibian, and Mammalian Species,* were as follows:

1. To study the feasibility of using mitogen-induced proliferation and cytotoxic T cell (CTL) immunoassays as biomarkers for immunomodulation in Tilapia.
2. To determine if immunoassays can be used to detect immunotoxicity in fish.
3. To delineate whether fish immunotoxicology can be used as a biomarker of pollution and serve as a sensitive indicator of effective management policies aimed at improving water quality using both field and lab studies.
4. To determine whether the proliferative response of B and T cells of *Bufo marinus* from polluted versus less polluted areas in Bermuda is different.
5. To study histological differences between the amphibians from polluted versus less polluted areas.
6. To determine the effects of TCDD exposure on the mouse fetus and neonate.

In summary, the overall goal of this study was to investigate the toxic effects of environmental pollutants using fish, amphibian, and mammalian systems as models. In the current study, we tested the central hypothesis that the immune system can serve as a sensitive biomarker to study the toxic effects of environmental pollutants in a wide range of species including fish, amphibians, and mammals.

Chapter 2: Laboratory and Field Studies on the Immune System in Fish: The Toxicity Induced by Environmental Pollutants.

Lisa M. Hudson Pélanne[1], Mitzi Nagarkatti[2], Don Orth[3], Vann Stancil[3], Bob Duncan[2], Prakash S. Nagarkatti[1]. [1]Department of Biology, [2]Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, [3]Department of Fisheries and Wildlife, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Abstract

The feasibility of using immune functions in fish as a biomarker of environmental pollution was studied. Tilapia was chosen as the model laboratory species. We identified mitogen-induced cell proliferation and cell-mediated cytotoxicity as two functional immunologic assays to study the immunotoxicity induced by 1,3-Bis (chloroethyl)-1-nitrosourea (BCNU). Tilapia lymphocytes responded to stimulation with only phorbol ester (PMA) in combination with calcium ionophore, but not to concanalvalin A (ConA), phytohemmaglutinin (PHA), or lipopolysaccarhide (LPS). The mitogen-induced proliferation assay was applied to fish harvested from various polluted and less polluted areas of the Roanoke River in Virginia. The various species of fish tested exhibited differential mitogen responsiveness. Unlike Tilapia lymphocytes that responded to mitogens at 37° C, the fish collected from the Roanoke River showed proliferative responses to mitogens at 28° C. Fish sampled from the more polluted North Fork of the Roanoke River exhibited enhanced mitogen responsiveness when compared to those tested from the less polluted South Fork of the Roanoke River. Finally, histological studies on the liver, ovary, skin, and spleen were done to determine if immune function is related to parasitic infection susceptibility. Together, the current study demonstrates that immunological perturbations in fish can serve as a biomarker for evaluating water quality.

Introduction

Fish represent the largest and most diverse group of vertebrates that live in a wide variety of habitats. Fish are particularly useful for the assessment of water-borne and sediment-deposited toxins where they may provide advanced warning of environmental pollution (Powers, 1989). For example, the incidence of hepatocyte carcinomas was found to be elevated in the English Sole (*Pleuronectes vetulus*) and was linked to excess polycyclic aromatic hydrocarbons in the sediments (Malins, 1985).

The fish immune system is well developed and comparable to the mammalian immune system, as it consists of both T and B cell-mediated immunity, constituting the cellular and humoral components (Wester, et al., 1994). However, fish do not have lymph nodes or bone marrow. In at least some species, the pronephros is an integrated part of the interrenal haemopoietic tissue (Wester, et al., 1994).

Catfish peripheral blood leukocytes (PBLs) respond to *in vitro* stimulation with phorbol ester (TPA) in conjunction with calcium ionophore leading to cell proliferation. This synergistic effect was stronger than all other mitogens and stimulated both T and B cells (Lin, 1992). The lymphoproliferative response of Spot (*Leiostomus xanthurus*) was different in fish sampled from sites with different levels of PAH contamination (Faisal, 1991). A genotoxic response related to the release of chemicals from the U. S. Department of Energy reservation in Tennessee was observed in sunfish in 1987 and 1988 (Shugart and Theodorakis, 1994). Lindane, an organochloride freshwater pollutant, suppresses the immune system in trout (Dunier and Siwicki, 1994). These data support the hypothesis that functional immunoassays, used to screen the immune system of fish, can serve as useful biomarkers to detect environmental pollution and possible health hazards.

The Roanoke River in Virginia has been named a critical natural resource by Roanoke City and County Planning Commissions. The advisory committees acknowledge that there is a direct link between land use and the health of the Roanoke River, and that the characteristics of lands adjacent to the Roanoke River, the uses, and scale of activity differ from jurisdiction to jurisdiction (Roanoke River Corridor Study Policy, Technical, and Citizens Advisory Committees, 1993). The upper Roanoke River Watershed (URRW) covers 1,500 km^2 in Montgomery, Roanoke, Botetourt, and Floyd counties, Virginia (Dickson, 1979). Urbanization is increasing in the URRW, especially in the cities of Roanoke, Salem, and the surrounding areas (Stancil, 2000). Conversion of forests to agriculture or development can change streams in several ways such as altered channel form, increased sedimentation, reduced abundance of woody debris, increased hydrologic variability, and increased temperature variability (Stancil, 2000).

In the current study, we investigated whether functional tests, used to screen the immune system of fish, can serve as a useful biomarker to assess environmental pollution. We demonstrated in the laboratory that administration of known immunosuppressive agents to the fish causes alteration of immune functions. We also gathered field data that suggest fish from polluted and non-polluted areas of the Roanoke River in Virginia exhibit different immune responses. These data suggest that immune function assays can serve as a useful tool to evaluate environmental pollution and quality of water based on land use data.

Materials and Methods

Fish. Tilapia (*T. mossambica*) for the laboratory studies were generously provided by the Department of Fisheries and Wildlife Aquaculture Center at Virginia Tech. Tilapia was chosen as the laboratory test species due to its availability as an aquaculture species. Proliferative and cytolytic assays were carried out on the tilapia in order to determine if these assays could be used as functional tests to screen the immune system of fish. They were housed in flow-through tanks at 26 ± 2° C and fed once per day *ad libitum*. Fish species from the Roanoke River were provided by Dr. Don Orth. The fish were caught by electroshock at the following sites: North Fork, South Fork, Wilson creek, Tinker creek, Upper Bottom creek, Upper Elliot creek, Wolf creek, Middle Back creek, Bradshaw creek, Middle North Fork, Middle Bottom creek, Bottom creek, and Elliot creek. The fish were anesthetized with methanesulfonate salt (MS222; Sigma, St. Louis, MO) and splenectomies were performed either on site and the spleens placed into ice cold medium or the fish were brought back to the lab on ice. All further manipulations were performed in a sterile hood environment.

BCNU Treatment. 1,3-Bis (chloroethyl)-1-nitrosourea (BCNU) was obtained from the National Cancer Institute (Bethesda, MD). BCNU was dissolved in minimal amounts of 70% ethanol or dimethylsulfoxide (DMSO) and then diluted with phosphate buffered saline (PBS) (Nagarkatti and Kaplan, 1985) for intraperitoneal injection of 30 mg/kg body weight into the fish on day 0. Fish were sacrificed 24 hours later.

Lymphocyte preparation. Splenectomies were performed and the spleen placed in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10 mM Hepes, 1 mM glutamine, 40 µg/mL gentamicin sulfate, and 50 µM 2-mercaptoethanol. Single-cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation at 1000 rpm for 10 minutes at 4º C. The splenocytes were subjected to density-gradient centrifugation using ficoll-hypaque (Sigma) to isolate the lymphocyte population from the erythrocytes (Nagarkatti, et al., 1990). Isolated lymphocytes were then further washed three times.

Total Cellularity. Lymphocytes prepared as described above were resuspended in medium. Twenty-five microliters of the single-cell suspension were added to 100 microliters of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under an inverted-phase contrast microscope.

Lymphocyte Proliferation. Proliferation assay was used to study the DNA synthesis (Rhile, et al., 1996) in fish lymphocytes. Varying cell concentrations from $6x10^5$ to $1.2x10^6$ cells per well in 0.2 mls of medium were stimulated with varying concentrations of mitogens, including phorbol ester (PMA) (5-20 ng/ml) and calcium ionophore (0.25-1 µM), phytohemmaglutinin (PHA) (12.5-50 µg/ml), concanalvalin A (ConA) (1-4 µg/ml), and lipopolysaccarhide (LPS) (12.5-50 µg/ml) (Sigma). The assay was run in a 96-well tissue culture plate for 24-62 hours at 37°C or 28°C. Six to eighteen hours before harvesting, the plates received 50µL of ^{3}H-thymidine. The cells were harvested using an automated cell-harvesting machine (Skatron,

Sterling, VA). The radioactivity was analyzed in a liquid scintillation counter (Tm Analytical, Elk Grove Village, IL). Results were expressed as radioactive counts per minute (CPM). In addition, in all experiments stimulation index was also calculated by dividing the CPM obtained in cultures receiving mitogens with the CPM seen in medium controls.

Cytotoxicity using ^{51}Cr-release assay. ^{51}Cr-release assay was used to evaluate the killing of tumor cells by natural killer (NK) cells in the spleen (McKallip, et al., 1995). Briefly, YAC-1 tumor cells (0.05×10^6 cells/ml) were labeled with 50 μCi ^{51}Cr by incubating at 37° C for 60 minutes. Various ratios of effector to target cells in triplicate were mixed in 96-well round-bottom plates (Costar, Cambridge, MA) and incubated at 37º C for 4 hours. Spontaneous release was measured by incubating ^{51}Cr-labeled cells alone, and total release was determined by incubating target cells with 0.1% sodium dodecyl sulfate (SDS). After 4 hours incubation, the supernatants were harvested and radioactivity measured using a gamma counter (Tm Analytical, Elk Grove Village, IL). Cytotoxicity was calculated as follows: % specific killing = E - S/T - S x 100, where E is experimental release, S is spontaneous release, and T is total release.

Histology. Liver, spleen, ovary, and skin of representative fish from several sites of the Upper Roanoke River Watershed were fixed in 10% formalin after harvesting and examined histologically using hemotoxylin and eosin stain.

Statistical Analysis. Tilapia were analyzed individually in several experiments. Each Roanoke River site was sampled once with a varying number of fish being caught per site. Spleens from 2-9 fish of approximate equal size were collected for each experiment and analyzed individually.

All experiments were run in duplicate or triplicate. Student's t test was used to compare proliferative and cytolytic results and $p < 0.05$ was considered to be significant.

Results

Cytotoxic and proliferative assays may serve as useful indicators of immune status in fish.

The tilapia lymphocytes demonstrated significant levels of cytotoxicity against YAC-1 tumor cells in a range-finding study using two fish (Figure 1). Next, we tested whether injection of BCNU, an anti-cancer drug and known immunosuppressive agent in mammals (Clary, et al., 1990) would suppress the immune response in fish. BCNU was able to induce suppression of cytolytic responsiveness at most effector: target cell ratios (Figure 2).

Tilapia were tested for their proliferative response to several mitogens including phorbol ester (PMA) and calcium ionophore, phytohemmaglutinin (PHA), concanalvalin A (ConA), and lipopolysaccarhide (LPS). Each mitogen was tested at various concentrations and at various lengths of exposure in culture. Data from a representative experiment is depicted in Figure 3. Tilapia exhibited a strong proliferative response to PMA and calcium ionophore, but not to other mitogens at the concentrations tested when compared to the medium control (Figure 3). Despite changing the culture conditions, including various incubation temperatures and concentrations of mitogens, we were unable to demonstrate a proliferative response in Tilapia using PHA, ConA, or LPS. In some experiments, PHA elicited a weak response (Figure 4). The response to PMA and calcium ionophore was dose-dependent with maximum stimulation occurring at 5 ng/ml PMA and 1 μM of calcium ionophore (Figure 5). It should be noted that Tilapia lymphocytes failed to respond to PMA or calcium ionophore when used separately (data not shown).

We next investigated the effects of BCNU-treatment on the ability of lymphocytes from Tilapia to respond to PMA and calcium ionophore. BCNU-treatment caused significant decrease in proliferative responsiveness of Tilapia lymphocytes (Figure 6). These data suggest that the

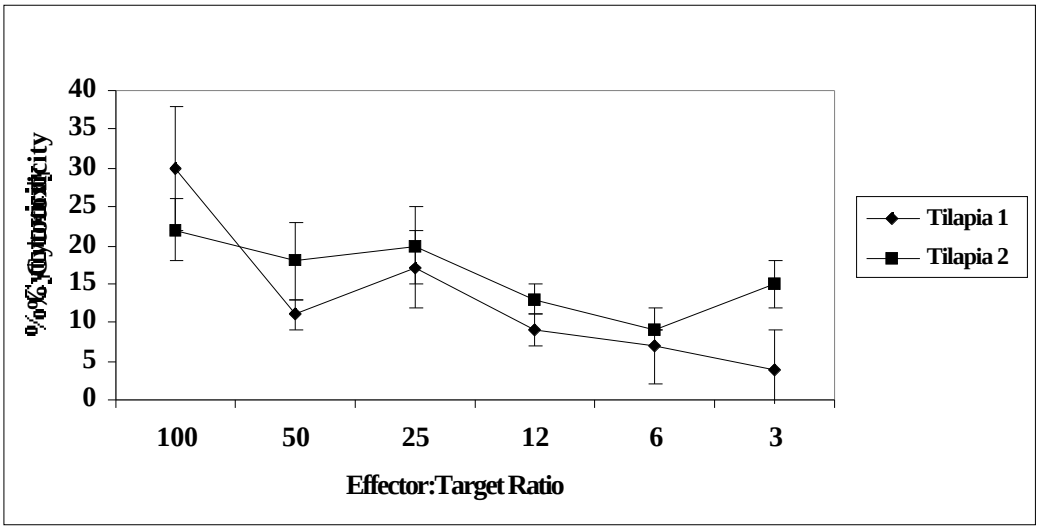


Figure 1. A range-finding study of cytolytic activity of tilapia lymphocytes against YAC-1 tumor targets. Purified lymphocytes were tested for cytotoxicity against ^{51}Cr-labeled YAC-1 target cells. Data represent mean +/- S.E.M. percent cytotoxicity derived from triplicate cultures. Tilapia 1 and 2 represent two individual fish tested.

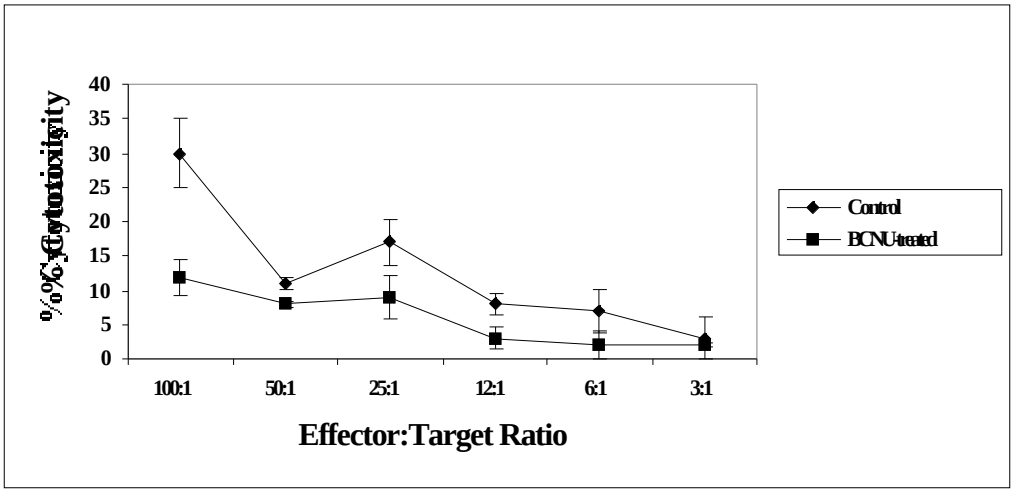


Figure 2. Effect of BCNU on cytolytic activity of tilapia lymphocytes. Tilapia were given a single intraperitoneal injection of either 30 mg/kg body weight of BCNU or the vehicle control. After 24 hours the spleens were harvested, lymphocytes purified, and percent cytotoxicity was measured against YAC-1 targets as described in Figure 1. Data represent mean +/- S.E.M. percent cytotoxicity derived from triplicate cultures from individual fish. A significant difference in percent cytotoxicity was detected in E:T ratios of 100:1, 25:1, 12:1, and 6:1, with $p<0.05$.

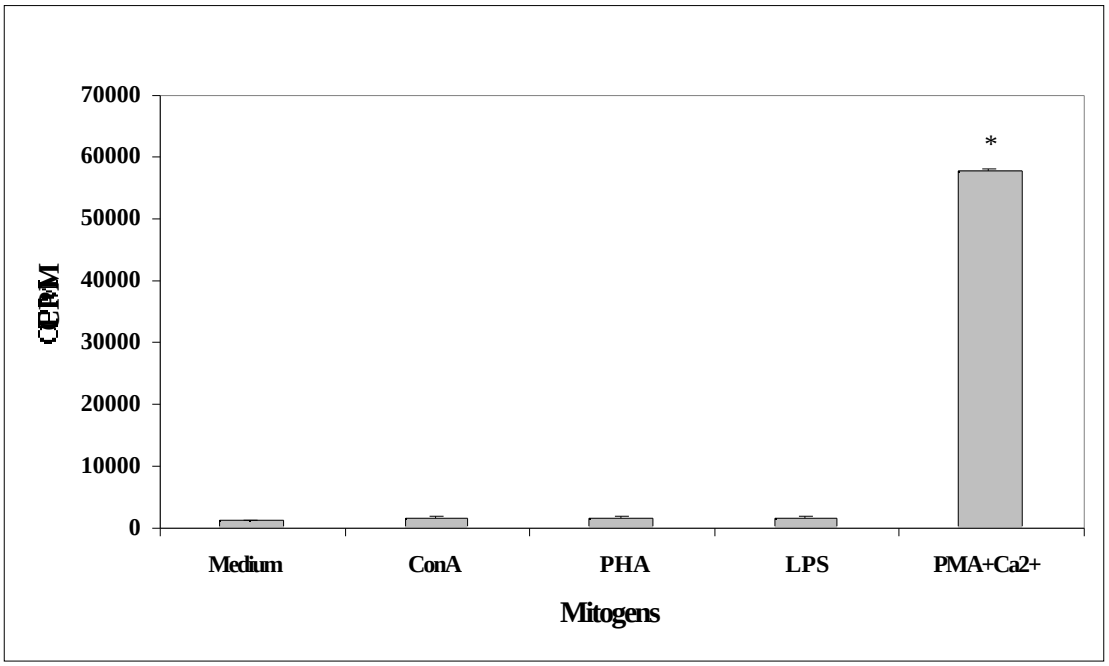


Figure 3. Proliferative response of tilapia lymphocytes to mitogens. Tilapia spleens were harvested, lymphocytes purified, and cultured *in vitro* with ConA (2 µg/ml), PHA (25 µg/ml), LPS (25 µg/ml), or PMA (10 ng/ml) and calcium ionophore (Ca2+) (0.5 µM). Mitogens were tested at several concentrations for an incubation period of 48 hours. Data represent mean +/- S.E.M. counts per minute (CPM) derived from triplicate cultures of an individual tilapia. Data shown is representative of in excess of three separate experiments. ($p<0.05$).

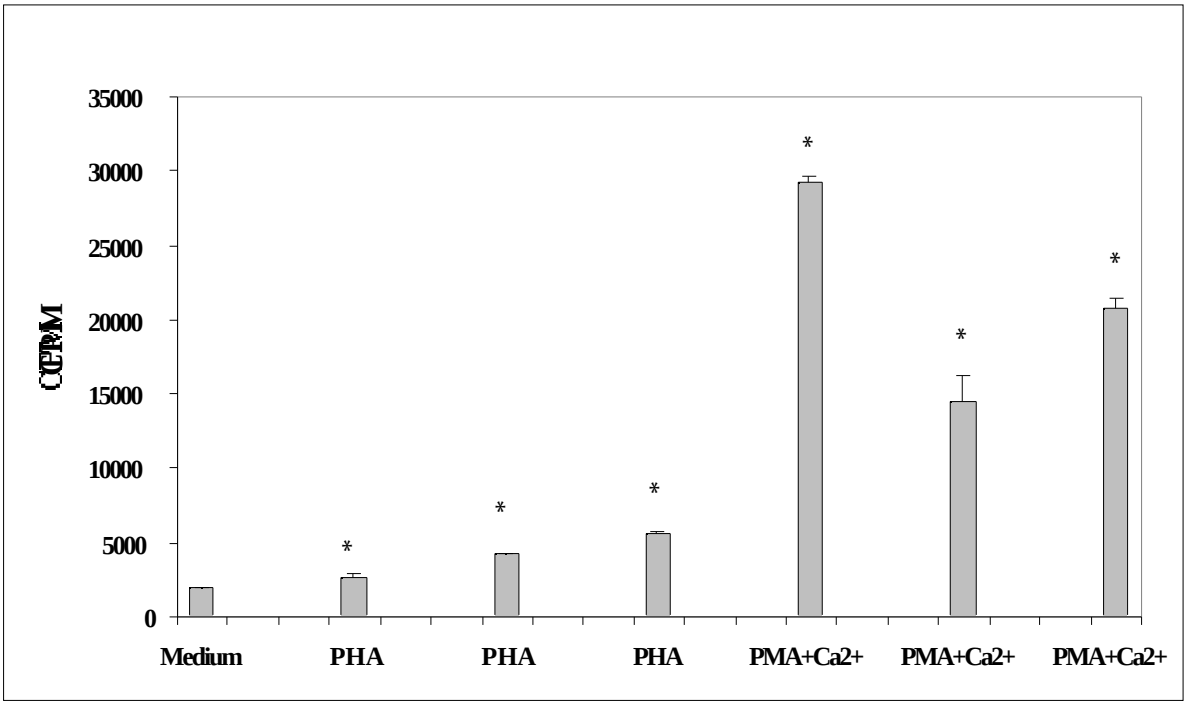


Figure 4. Proliferative response of tilapia lymphocytes to PHA and PMA with calcium ionophore. Tilapia spleens were harvested, lymphocytes purified, and cultured *in vitro* with various concentrations of PHA or PMA with calcium ionophore ($Ca2^{+}$). The concentrations of PHA used were (left to right) 50 µg/ml, 25 µg/ml, and 12.5 µg/ml. The concentrations of PMA with $Ca2^{+}$ from left to right were 20 ng/ml PMA with 1 µM $Ca2^{+}$, 10 ng/ml PMA with 0.5 µM $Ca2^{+}$, and 5 ng/ml PMA with 0.25 µM $Ca2^{+}$. Data represent mean +/- S.E.M. counts per minute derived from triplicate cultures. Data shown is representative of in excess of three separate experiments. ($p<0.05$).

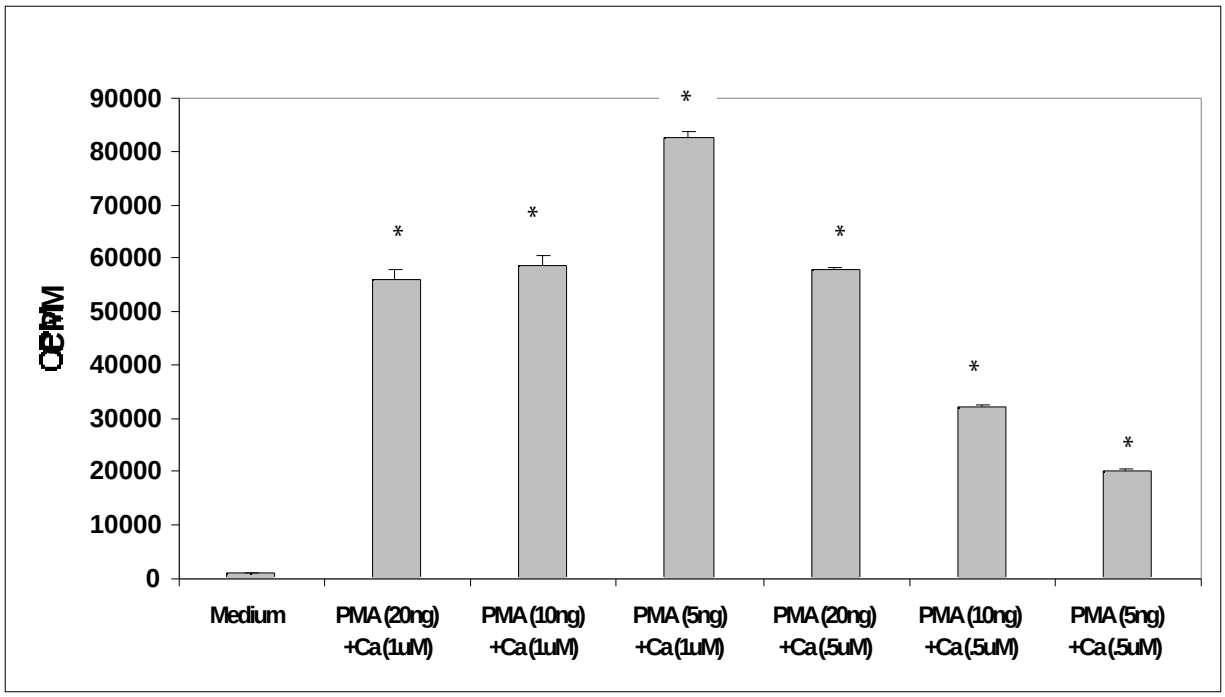


Figure 5. Proliferative response of tilapia lymphocytes to PMA and calcium ionophore (Ca) is dose dependent. Tilapia spleens were harvested, lymphocytes purified, and cultured *in vitro* with various concentrations of PMA and calcium ionophore. Lymphocytes were cultured in vitro for 24 hours. Data represent mean +/- S.E.M. counts per minute derived from triplicate cultures. Data shown is representative of in excess of three separate experiments. ($p<0.05$).

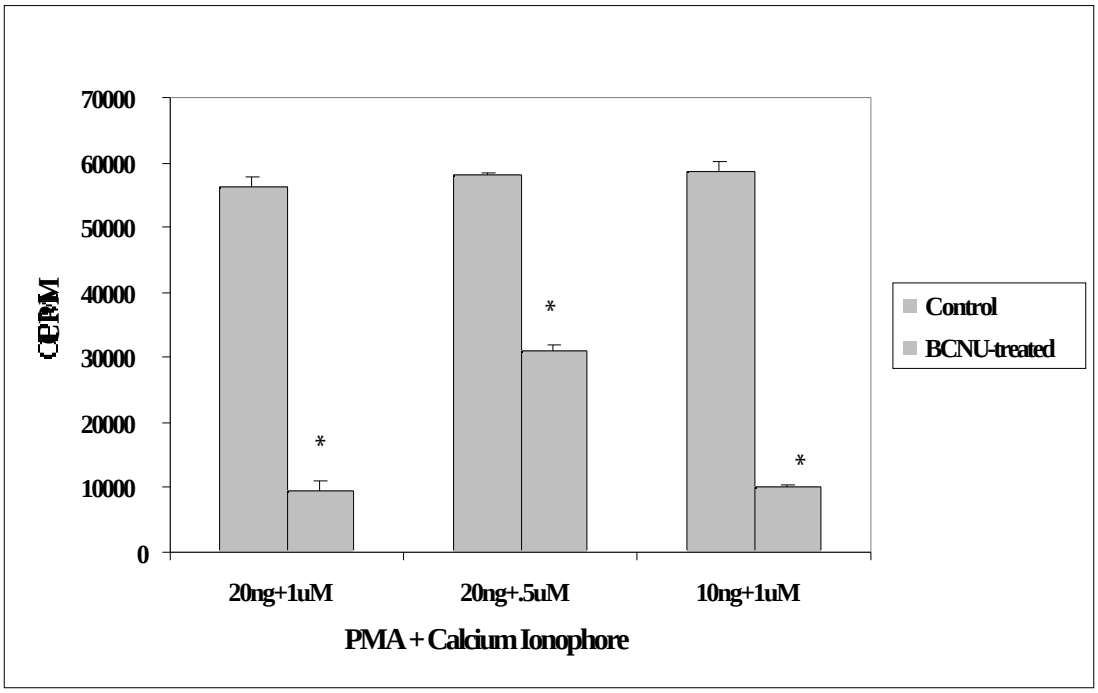


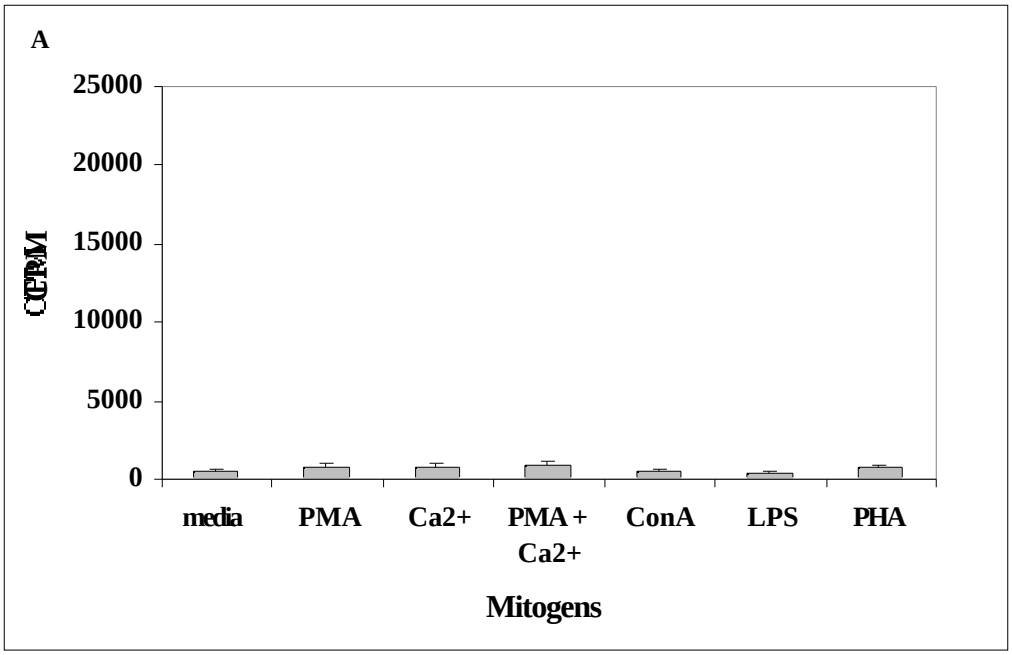
Figure 6. Proliferative response of tilapia lymphocytes to PMA and calcium ionophore following treatment with BCNU. A single intraperitoneal injection of 30 mg/kg body weight BCNU was given on day 0. After 24 hours the spleens were harvested, lymphocytes were isolated and cultured for 48 hours *in vitro* in the presence of the mitogens. Data represent mean +/- S.E.M. counts per minute derived from triplicate cultures from a representative experiment. ($p<0.05$).

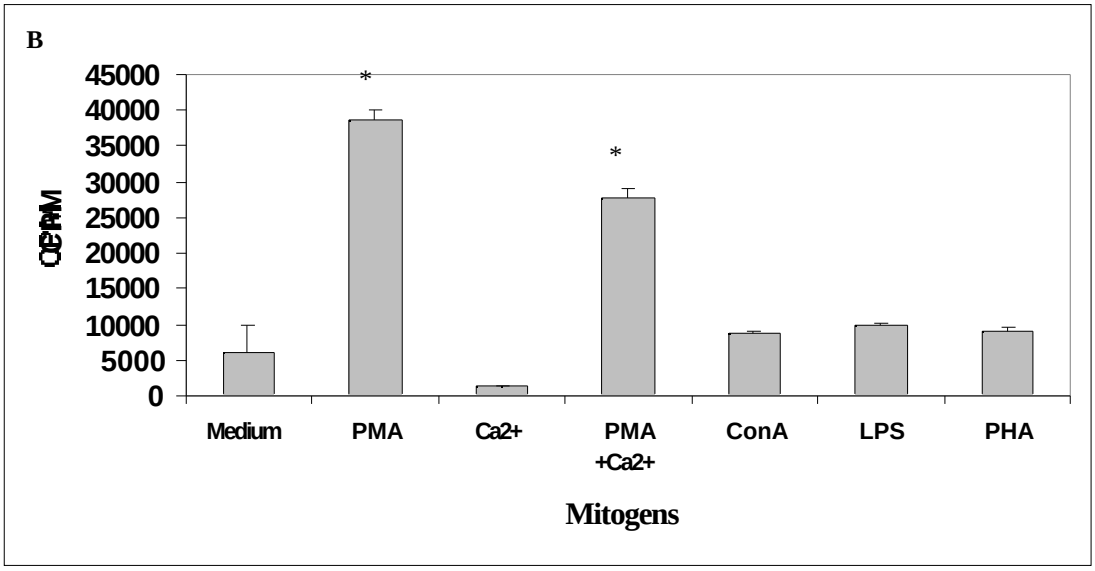
fish cytolytic and proliferative assays are sensitive tools able to detect immunosuppression caused by chemical exposure.

Temperature plays a role in the ability of fish lymphocytes to proliferate *in vitro*.

In the field study, several fish species were collected from various sites along the Roanoke River. The sites spanned across a study area that encompasses 575 square miles of the Roanoke River watershed. The spleens were collected on site and transported to the lab for immediate assessment under aseptic conditions, or fish were transported to the lab alive where the spleens were then harvested. The spleen cells were screened for their ability to respond to various B cell and T cell mitogens. No proliferative response occurred after culture of cells from white sucker at 37° C (Figure 7A). However, the lymphocytes exhibited a significant response after culture at 28° C (Figure 7B). Also, unlike Tilapia lymphocytes, cells from white sucker responded to stimulation with PMA alone but not to calcium ionophore. In fact, combination of PMA and calcium ionophore was less stimulatory than PMA alone (Figure 7B). This was more evident using lymphocytes from the northern hogsucker, which were stimulated by PMA alone but not by PMA and calcium ionophore (Figure 8). Lymphocytes from northern hogsucker responded to other mitogens, including LPS and PHA but not ConA (Figure 8).

Proliferative responsiveness of lymphocytes to mitogens from fish collected at North Fork and South Fork of the Roanoke River.





Mitogens

Figure 7. Proliferative response of white sucker lymphocytes cultured at different temperatures. The fish were caught from the Roanoke River by electroshock, spleens were harvested and lymphocytes isolated. Lymphocytes were cultured for 38 hours *in vitro* with ConA (2 µg/ml), PHA (25 µg/ml), LPS (25 µg/ml), PMA (10 ng/ml), or calcium ionophore (Ca2+) (0.5 µM) at 37°C (A) or 28°C (B). Data represent mean +/- S.E.M. counts per minute derived from triplicate cultures. Data shown is from an individual fish representative of three fish tested. ($p<0.05$).

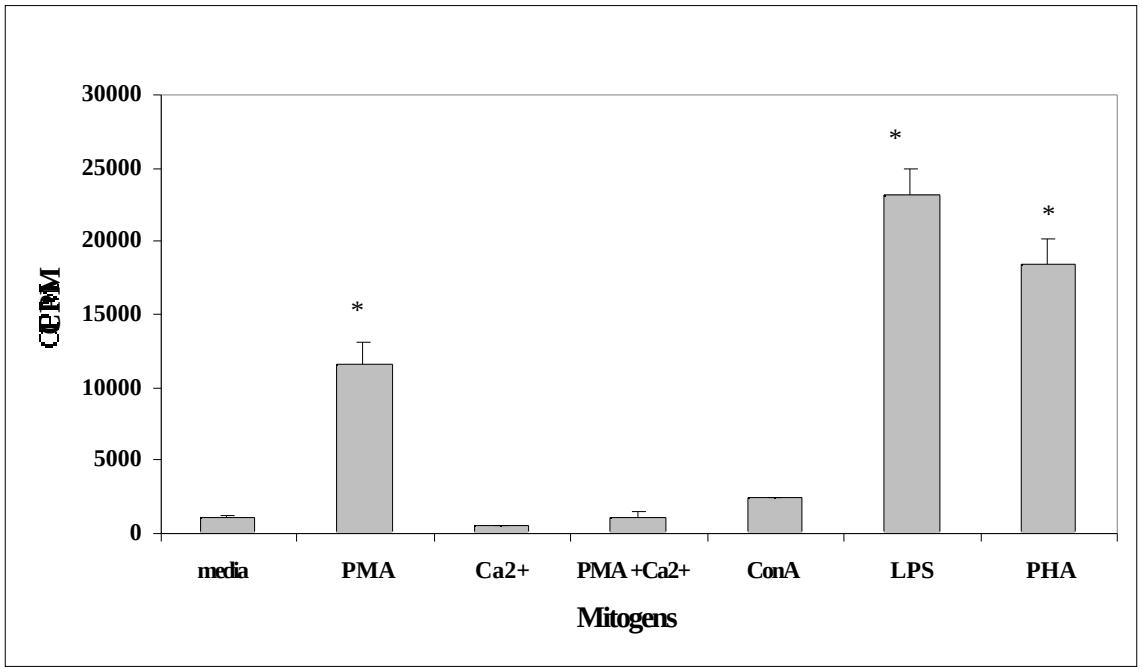


Figure 8. Proliferative response of northern hogsucker lymphocytes cultured at 28° C. The fish were caught from the Roanoke River by electroshock, spleens were harvested and lymphocytes isolated. Lymphocytes were cultured for 38 hours with mitogens. Data represent mean +/- S.E.M. counts per minute (CPM) derived from triplicate cultures from an individual fish. ($p<0.05$).

Based on land use, the North Fork of the Roanoke River is considered to be more polluted than the South Fork of the Roanoke River (Stancil, 2000). We therefore sampled fish from these areas and compared their immune functions. Fish collected from the North Fork and South Fork of the Roanoke River exhibited different responses to mitogens. In Figure 9, nine northern hogsuckers collected from the South Fork all exhibited a stimulation index of 2.7 or less when stimulated with PMA, ConA, LPS, and PHA. However, six northern hogsuckers collected from the North Fork of the Roanoke River exhibited higher stimulation indexes when stimulated with the same mitogens. A particularly strong proliferative response was seen in four of the fish when cultured with LPS, with a stimulation index of up to 16.2. These data suggest that the fish from the North Fork of the Roanoke River were able to mount a stronger lymphocyte proliferative response to certain mitogens when compared to similar fish from the South Fork of the Roanoke River.

Histological analysis of fish caught from the Roanoke River.

The organs of the fish were studied for histological anomalies. Anomalies noted included internal and external parasites, granulomas of the liver and spleen, and tumor-like growths. Parasites identified include leeches (Hirudinea), trematodes, coccidian parasites, cestodes, microsporidia, myxosporidia, and nematodes. Almost all of the fish from the study areas exhibited the external parasitic life stage known as blackspot disease.

In the North Fork of the Roanoke River, internal parasites (trematodes and cestodes) were identified in five of the nine central stonerollers studied. Trematodes were found in the liver, spleen, and skeletal muscle tissue of the fish. Cestodes were identified in the spleen. A tumor-

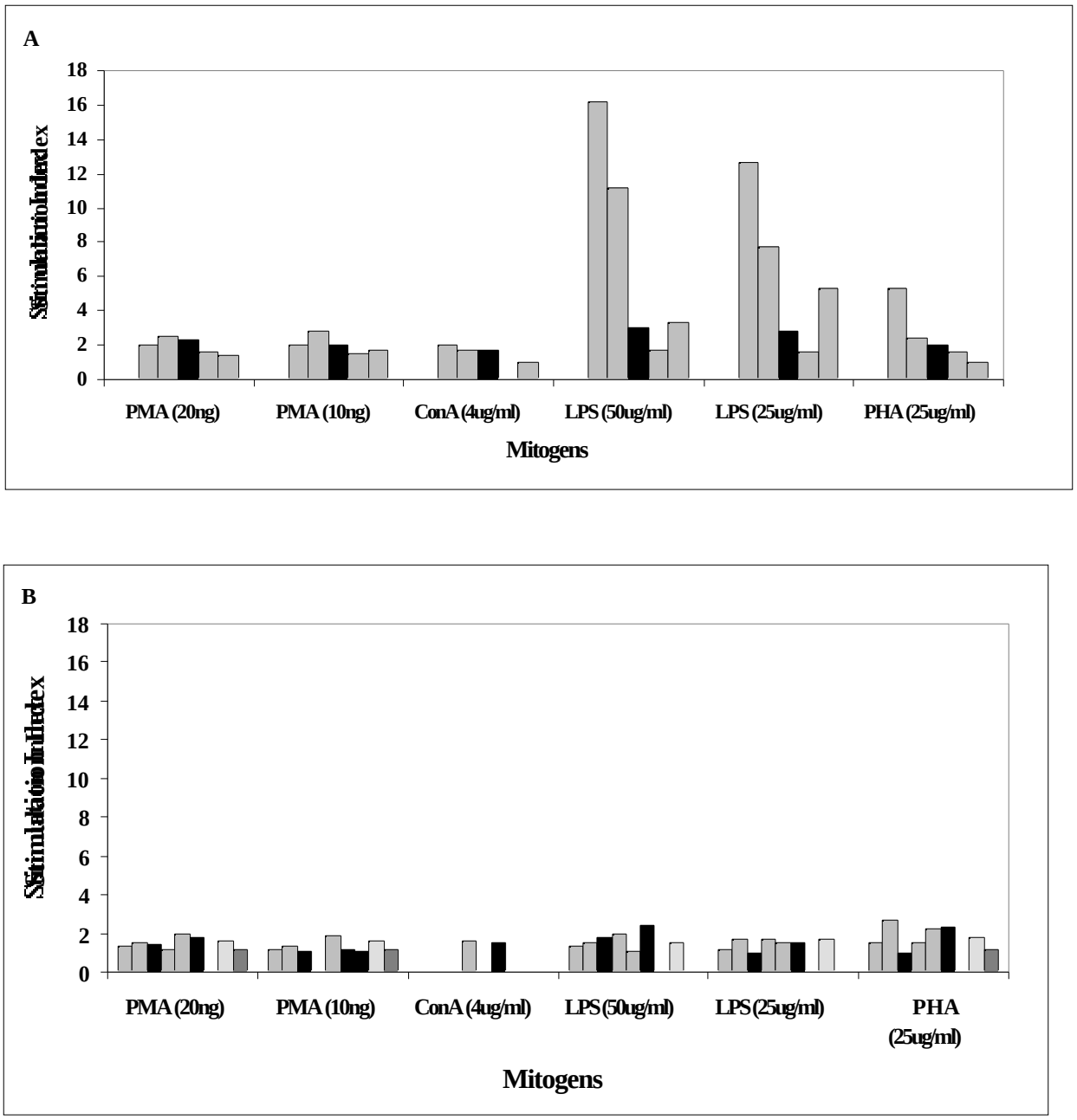


Figure 9. Fish from the North Fork and South Fork of the Roanoke River exhibit different responses to mitogens. Northern hogsuckers were caught from the North Fork (n=5) (A) and South Fork (n=9) (B) of the Roanoke River by electroshock. The fish spleens were harvested and lymphocytes isolated. The lymphocytes were then cultured *in vitro* with ConA, PHA, LPS, or PMA. Stimulation index is indicated on the Y-axis. Each vertical bar represents data from an individual fish. Data represent mean stimulation index derived from triplicate cultures. Stimulation index was derived by dividing the counts per minute of cultures incubated with mitogens by the mean counts per minute in cultures incubated with medium alone.

like external growth was identified on one of the fish collected. In the South Fork of the Roanoke River, only one internal trematode was identified in the twelve central stonerollers studied. These data suggest that the fish from the North Fork were more susceptible to parasitic infection when compared to the fish collected from the South Fork.

In Upper Bottom Creek, three species of fish were collected for study: one bluehead chub, five crescent shiners, and six central stonerollers. Trematodes were identified in the skin of eight of the twelve fish studied. One cestode was identified in the bile ducts of a central stoneroller. Coccidian parasites were identified in the spleen of two central stonerollers and one crescent shiner. Granulomas were identified in the liver of one crescent shiner. Only one crescent shiner of the twelve fish studied exhibited no histological anomalies.

Seven fish were caught for study from Upper Elliot Creek, one central stoneroller, one bluehead chub, and five mountain redbelly dace. Only one mountain redbelly dace out of the seven fish studied exhibited any anomalies, a splenic granuloma.

The fifteen fish caught for study from Wolf Creek included crescent shiner, central stoneroller, mountain redbelly dace, creek chub, and bluehead chub. One crescent shiner, three central stonerollers, and one of three creek chubs exhibited no histologic anomalies. The other two creek chubs exhibited trematodes in the liver and/or granulomas in the spleen. Coccidian parasites in the spleen, myxosporidian in the muscle, and granulomas in the spleen, liver, and muscle were identified in the mountain redbelly dace. All of the bluehead chub exhibited granulomas of either the spleen, liver, muscle, or ovary.

One creek chub, one rosyside dace, and five bluehead chub were studied from Middle Back Creek. Microsporidia were identified in the ovary of the creek chub. No histological

anomalies were noted in the rosyside dace. Granulomas were identified in the muscle and spleen of two of the bluehead chub, whereas the other three were free of any anomalies.

Seven fish were studied from Bradshaw Creek including a redbreast sunfish, two bluehead chub, and four rosyside dace. Granulomas were identified in the muscle of the redbreast sunfish, muscle of one bluehead chub, and liver of one rosyside dace. No other anomalies were noted.

Four crescent shiners, four torrent suckers, and two central stonerollers were studied from Middle North Fork of the Roanoke River. Myxosporidia was identified in the muscle and a nematode in the pancreatic tissue of one crescent shiner. One torrent sucker exhibited granuloma in the liver. No other anomalies were noted.

Five crescent shiners and five bluehead chub were caught for study from Middle Bottom Creek. Anomalies were identified in all five of the crescent shiners including myxosporidia in the muscle, trematodes in the muscle, cestodes in the liver, coccidian in the spleen and pancreas, and numerous granulomas in the spleen and liver. Four of the five bluehead chub exhibited anomalies including trematodes in the skin, myxosporidia in the spleen, and granulomas in the liver and spleen.

Ten fish were caught for study from Bottom Creek including two smallmouth bass, two white suckers, two torrent suckers, and four central stonerollers. Trematodes were identified in the muscle of one of the smallmouth bass, and nematodes were identified in the liver of the other. Proliferative branchitis was noted in both of the smallmouth bass. No anomalies were noted in the white suckers. Granulomas were identified in the spleen or muscle of the two torrent suckers, and trematodes were found in the skin of one of the torrent suckers. All four of

the central stonerollers were infected with parasites including trematodes in the skin and coccidian in the spleen.

Eight fish were caught for study from Elliot Creek including one white sucker, one cutmount, three torrent suckers, and three central stonerollers. There were no anomalies identified in the white sucker, cutmount, torrent suckers, and one of the central stonerollers. The other two stonerollers exhibited either an external parasitic infection of Hirudinea or a granulomous liver.

Of these tributaries, Bradshaw Creek, Middle Bottom Creek, Lower Bottom Creek, and South Fork are considered to be the most healthy based on their land cover being primarily forested (Stancil, 2000). Upper Elliot Creek, Elliot Creek, Wilson Creek, Middle North Fork, and North Fork are in agricultural areas and some have poor riparian zones (Stancil, 2000). These conditions may lead to leaching of agricultural chemicals and pollutants easily into the stream habitat. Wolf Creek, Tinker Creek, and Middle Back Creek are in areas with more urban development and are considered to be the least healthy (Stancil, 2000).

Discussion

Proliferative and cytolytic assays were carried out on the tilapia lymphocytes in order to determine if these assays could be used as functional tests to screen the immune system of fish. In the current study, we demonstrated that tilapia exhibited significant levels of cytotoxicity against YAC-1 tumor cells, and that the cytolytic response could be suppressed following treatment with BCNU. The role of nonspecific cytotoxic cells, including natural killer (NK) cells, in fish is not well understood or characterized (Graves, et al., 1984, Heath, 1995). YAC-1 tumor target cells were chosen because they are NK-cell sensitive. The ability of the tilapia lymphocytes to lyse the YAC-1 targets suggested the presence of NK cells in the spleen of the tilapia. Future studies of NK activity in tilapia may be useful to study anti-tumor immunity in the fish immune system, as NK cells are involved in nonspecific killing of tumor cells as well as virally-infected cells.

We also demonstrated that tilapia lymphocytes respond dose-dependently to PMA and calcium ionophore but not to ConA, PHA, or LPS. Other studies report that fish lymphocytes do respond to mitogenic stimulation by Con A, PHA, and LPS (Faisal, et al., 1991). The temperature range in the studies reported here may have been prohibitive and explain why those mitogens did not elicit a response. Treatment of fish with BCNU caused suppression in mitogen responsiveness, as evident by the decreased proliferative activity noted after BCNU treatment when compared to the controls. The ability to detect immunotoxicity in the tilapian immune system suggests that tilapia is a useful species for studying effects of pollutants or drugs in a laboratory setting. Fish may serve in lieu of mammalian studies, especially in the case of aquatic pollutants, where fish would naturally be the first vertebrates affected. Fish immunosuppression

may well serve as a biomarker of potential toxins in the human environment, and is particularly useful for the assessment of water-borne and sediment deposited toxins where it may provide advanced warning of the potential danger of new chemicals (Powers, 1989).

In the field studies, we found that temperature plays an important role in the ability of fish lymphocytes to proliferate *in vitro*. Collazos and colleagues (1995) report that environmental temperature changes affect the immune system of fish. While the tilapia lymphocytes were able to respond to the mitogens at 37º C, the fish collected from the Roanoke River were not. This is probably because tilapia are a tropical species whereas the fish collected from the Roanoke River are temperate species. We found the ideal temperature at which fish from the Roanoke River responded to mitogens was 28º C; however, not all of the fish tested were able to proliferate ideally at this temperature, and the ideal temperature may vary according to season and fish species.

Unlike the tilapia, fish collected from the Roanoke River responded to ConA, LPS, PHA, and PMA alone. Thus, calcium ionophore was not necessary to induce lymphocyte proliferation. This is in contrast to the mammalian lymphocytes, which fail to respond to PMA stimulation alone and need calcium ionophore (Kakkanaiah, et al., 1990). Furthermore, mammalian lymphocytes respond to stimulation with calcium ionophore alone, unlike the fish lymphocytes (Kakkanaiah, et al., 1990). The variation in mitogen responsiveness within and across species makes field studies difficult to compare with those carried out in the laboratory. One solution may be to study a single species from the field at a single time of year, or to attempt to acclimate and keep the single species in a constant laboratory environment for a period of time.

We found that the fish from the North Fork and South Fork areas of the Roanoke River responded differently to mitogens. The North Fork is considered to be more polluted when

compared to the South Fork due to the type of land use associated with the surrounding areas (Stancil, 2000). Land cover in the North Fork is forested with agriculture, primarily with livestock grazing, occurring in lower elevations near streams, while land cover in the South Fork is primarily forested (Stancil, 2000). We found that the fish collected from the South Fork had little or no proliferative response to mitogens, while the fish collected from the North Fork exhibited stronger proliferative response to the various mitogens. In order to accommodate for the variation in response, we looked at the stimulation index of the proliferative response, which compares the individual fish's proliferative response to the mitogens and to the medium alone.

Considering the fact that the North Fork of the Roanoke River is more polluted when compared to the South Fork, we expected the fish from the North Fork to mount a decreased immune response when compared to those from the South Fork. However, the results were surprisingly opposite inasmuch as fish from the North Fork of the Roanoke River mounted a stronger response to certain mitogens such as LPS. This may be because the fish from the North Fork were heavily infected with parasites thereby activating the B lymphocytes. Such activated cells upon restimulation may have exhibited a stronger secondary response. Many antigens from infectious agents are known to activate a large proportion of lymphocytes and are designated as "superantigens." (Roitt, et al., 1998). Further studies in the laboratory should help resolve this observation. It is interesting to note that the fish from the North Fork of the Roanoke River responded to T cell mitogens to the same extent as the fish from the South Fork of the Roanoke River.

Studies show that the histopathological changes in animal tissues are indicators of prior exposure to environmental stressors (Hinton, et al., 1990). The histological portion of this study focused on parasitic infection. The rates of parasitic infection and anomaly ranged from 8.33%

in the South Fork to 91.67% in Upper Bottom Creek. Interestingly, these two tributaries of the Roanoke River are both classified as healthy, with the land use being described as primarily forested (Stancil, 2000). The three broad classifications of land use defined in this study as forested, agricultural, or disturbed (Stancil, 2000) did not correlate with the incidence of parasitic infection based on the small cross-section of fish sampled for histological examination. Further studies and increased samples are recommended for further analysis.

There is growing concern about the health status of aquatic species in relation to environmental pollution. Previous studies have suggested that various functional aspects of fish may serve as an excellent biomarker for pollution. These studies indicate that toxic effects on the immune system (immunotoxicity) may serve as an excellent biomarker to monitor pollution and water quality. In addition, this study emphasizes the effects of land use and the resulting habitat changes and pollution, which affect stream health and consequently, fish health.

Chapter 3: Role of Apoptosis in TCDD-Induced Toxicity in Perinatally Exposed Neonates.

L M Hudson Pélanne[1], M Nagarkatti[2], P S Nagarkatti[1]. [1]Department of Biology, [2]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia.

Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental contaminant considered to be the most toxic chemical ever created. In previous studies, TCDD was shown to induce apoptosis, contributing to thymic atrophy in C57BL/6 mice. In the current study, we tested the hypothesis that prenatal administration of TCDD leads to immunotoxicity due to induction of apoptosis in fetal and neonatal thymocytes. To this end, 0, 0.1, 1, or 5 micrograms per kilogram body weight of TCDD was administered via a single intraperitoneal injection to pregnant mice on gestational day 14. The thymocytes were studied on postnatal days 2, 4, 7, 14, and 21. We found a decrease in thymic cellularity following TCDD treatment on postnatal days 14 and 21. Apoptosis was detected in freshly isolated thymocytes exposed to TCDD in 25% of 2-day old pups using the TUNEL method. Furthermore, TCDD caused an increase in percent apoptosis in thymocytes of 2 and 4 day old pups following 24-hour *in vitro* culture. Little or no apoptosis was detected in 7, 14, and 21 day old pups following 24 hour culture, however an increase was detected after 12 or 18 hours of *in vitro* culture. TCDD treatment did not alter the proportion of T cell subsets in 2 day old pups. CD3, αβ-TCR, CD44, IL-2R, and J11d markers showed little change following 1 μg/kg TCDD exposure in 2 day old pups. However, the percentage of T cells expressing these markers was significantly decreased when exposed to the 5 μg/kg dose of TCDD. Together, these data suggest that apoptosis may play a significant role in TCDD-induced immunotoxicity seen in perinatally exposed mice.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxide (TCDD) is an environmental contaminant considered to be the most toxic chemical ever created. It is formed as a byproduct in the manufacture of products from chlorinated phenols or during combustion of chlorinated materials. The immune system is highly sensitive to TCDD and therefore this contaminant has raised significant health concerns (Holsapple, 1991). Recent studies show that TCDD treatment triggers apoptosis in thymocytes of adult C57BL/6 wild type mice (Kamath, et al., 1997). Also, *lpr/lpr* mice deficient in Fas, and *gld/gld* mice, which have defective Fas ligand (FasL), two molecules involved in apoptosis, were more resistant to TCDD-induced thymic atrophy (Rhile, et al, 1996, Kamath, et al., 1999). These data suggested that TCDD may upregulate Fas-FasL interactions thereby triggering apoptosis. Further studies revealed that TCDD increased the expression of FasL but not Fas in the thymus (Kamath, et al., 1999). Apoptosis is a type of cell death characterized by DNA fragmentation into oligonucleosomal-length fragments and widespread blebbing of the nuclear and plasma membranes, inducing cell death. Perinatal exposure to TCDD induces thymic atrophy in all species tested thus far and occurs at doses that are well below those that cause maternal and fetal toxicity (Birnbaum, 1991). Also, following perinatal exposure, the immunotoxic effects persist into adulthood (Gehrs, et al., 1997).

In the current study, we tested the hypothesis that perinatal exposure to TCDD leads to immunotoxicity due to induction of apoptosis in thymocytes. Our results suggested that perinatal exposure to TCDD induces apoptosis in the thymus of the pups, which persists up to three weeks after birth. This toxicity leads to lower thymic cellularity in older pups as a result of the

apoptosis. The ability of TCDD to induce apoptosis in the thymus may alter T-cell differentiation.

Materials and Methods

Mice. Adult, female, pregnant C57BL/6 mice were purchased from the National Institute of Health (Bethesda, MD). The pregnant females were given 0.1, 1, 5, or 10 micrograms per kilogram body weight of TCDD or the vehicle, corn oil, via a single intraperitoneal injection on gestational day 14. Dosing occurred on 11 different occasions and each dose was given a minimum of three times. The pups were kept with their mothers until sacrifice. The mice were housed in polyethylene cages containing wood shavings and given rodent chow and water *ad libitum*. Pups were sacrificed on days 2, 4, 7, 14, and 21 for analysis of apoptosis and thymic cellularity.

TCDD Exposure. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was generously donated by Dr. K. Chae of NIEHS, Research Triangle Park, NC and stored at -20° C. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone as previously described (Rhile, et al., 1996). Mice were administered various doses of TCDD or the vehicle by a single intraperitoneal injection.

Lymphocyte Preparation. Thymectomies were preformed on euthanized mice and the thymus placed in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% or 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10mM Hepes, 1mM glutamine, 40 μg/mL gentamicin sulfate, and 50 μM 2-mercaptoethanol. Single-cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati,

OH) and kept on ice. Cells were pelleted by centrifugation. Isolated lymphocytes were then further washed three times.

Total Cellularity and Percent Viability. Lymphocytes prepared as described above were resuspended in medium. Twenty-five microliters of the single-cell suspension were added to 100 microliters of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under an inverted-phase contrast microscope.

Apoptosis. Apoptosis was determined using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, commonly referred to as TdT-mediated nick end labeling, or TUNEL technique, as described elsewhere (Kamath, et al., 1997, 1999). The cells (5×10^6) were washed twice with medium containing phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cells were again washed with PBS, permeabilized on ice for 2 minutes and incubated with FITC-dUTP (Boehringer Mannheim, Indianapolis, IN) for one hour at 37º Celsius. Fluorescence of the cells was measured on a Coulter Epics V flow cytometer.

Detection of Phenotypic Markers on Thymocytes. One million thymocytes from TCDD or vehicle-treated mice were stained with fluorescein-conjugated monoclonal antibodies to CD3 (hamster IgG), CD4 (rat IgG2a), CD8 (rat IgG2a), CD44 (rat IgG2b), αβ−TCR (ratIgG), IL-2R (IgG2b), or J11d (rat IgM) (Pharmingen, Torregana, CA). The cells were incubated with the antibodies for 30 minutes on ice and then washed twice with PBS. Cells were then fixed in 1% paraformaldehyde. Fluorescence of the cells was measured on a Coulter Epics V flow

cytometer. Negative controls consisted of cells that were not stained with antibodies and represented autofluorescence (Kamath, et al., 1997, 1999).

Statistical Analysis. Each experiment was repeated at least twice to confirm the apoptosis results. Statistical analysis was completed by the Statistical Consulting Center at Virginia Tech using Bayesian statistical methods.

Results

Thymic Cellularity after Prenatal Exposure to TCDD

Pregnant C57BL/6 mice were treated with 0.1, 1, 5, or 10 μg/kg body weight of TCDD or the vehicle control on gestational day 14 via a single intraperitoneal injection. No pups that received the 10 μg/kg body weight dose of TCDD survived until postnatal day 2 to be evaluated. On postnatal days 2, 4, 7, 14, and 21, the pups were sacrificed and the organs harvested. The total number of viable thymocytes was determined by trypan blue dye exclusion. Pups were tested individually and the mean values were calculated for analysis. Three to eight pups were evaluated in each experiment and all experiments were repeated at least twice. As expected, the thymic cellularity increased in vehicle controls from 2-21 postnatal days (Figure 1). In TCDD exposed pups, a similar pattern was seen except that the cellularity was significantly lower at 14 and 21, but not 2-7 postnatal days. TCDD-induced decrease in thymic cellularity on days 14 and 21 was not dose-dependent. This may be because the pups continue to get exposed to TCDD postnatally via the mother's milk (Luster, et al., 1980). Thus, there could be slight variation in exposure to TCDD based on the amount of milk consumed by the pups.

Detection of Apoptosis in Thymocytes.

To address whether apoptosis was a possible mechanism for the decreased thymic cellularity in the pups, we used the TUNEL method to detect the broken strands of DNA that characterize apoptotic cell death. Apoptosis is difficult to detect *in vivo* due to active phagocytic

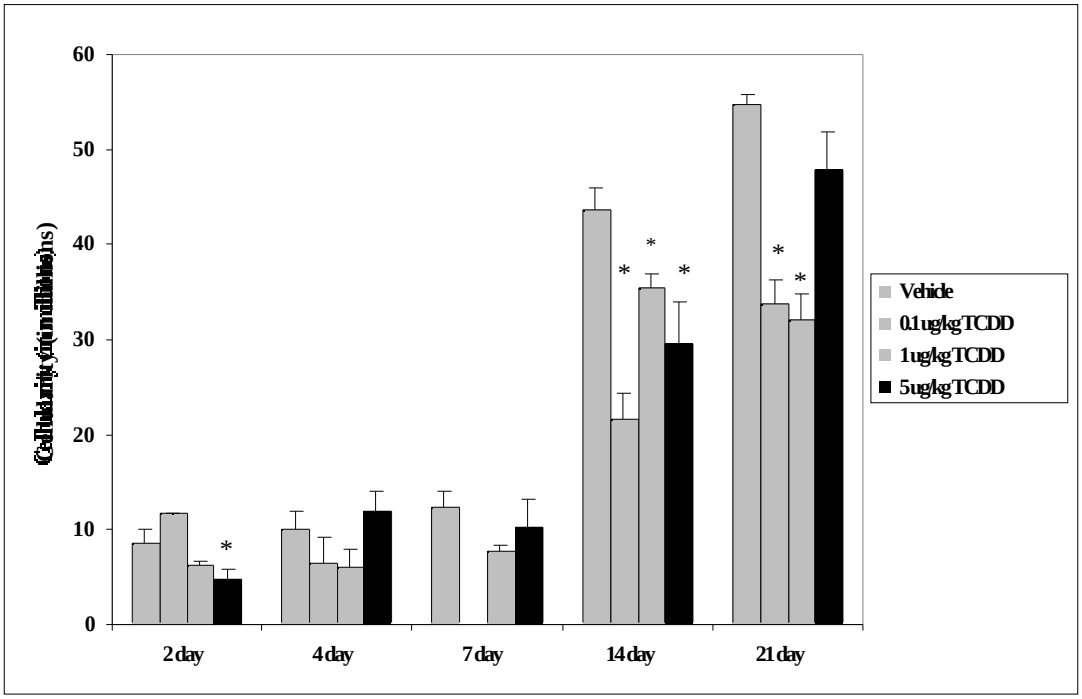


Figure 1. Effect of TCDD-treatment on thymic cellularity in 2-21 day old pups. Pregnant mice were treated with various doses of TCDD or the vehicle via a single intraperitoneal injection on gestational day 14. Thymectomies were performed on pups on postnatal days 2, 4, 7, 14, and 21. Thymic cellularity was determined using the trypan blue dye technique. Data represent mean cellularity (n=3 to 8) +/- S.E.M. Asterisks represent data found to be significant when compared to the vehicle controls ($p<0.05$).

cells in the thymus (Kamath, et al., 1997); however, in 25% of the experiments involving TCDD-exposed 2 day old neonates, we were able to detect apoptosis in freshly isolated thymocytes (Figure 2). Previous studies show that thymocytes from adult mice exposed *in vivo* to TCDD, upon *in vitro* culture, exhibit increased levels of apoptosis (Kamath, et al., 1997). To investigate this possibility in the pups, we cultured the TCDD-exposed thymocytes with medium *in vitro* and studied the cells for apoptosis.

Following 24-hour culture, we were able to detect an increase in percent apoptosis in the TCDD-treated 2, 4, and 7 day old neonates when compared to the vehicle-treated neonates (Figure 3). Little or no apoptosis was detected after 24-hour culture in the 14 day old pups; however, when cultured for a lesser amount of time, such as for 12 or 18 hours, apoptosis was detected using the TUNEL method.

Effect of TCDD on T cell subsets in the thymus of perinatally-exposed mice.

We next looked at the T cell subsets in the thymus of the pups. Thymocytes of TCDD and vehicle-treated pups were isolated and stained with FITC-labeled anti-CD4 and PE-labeled anti-CD8 mAbs and studied with flow cytometry. There were no significant changes in the T cell subsets of TCDD-treated pups when compared to the vehicle-treated pups, suggesting that TCDD toxicity affects all T cell subsets (Figure 4).

Phenotypic Changes in Thymocytes of Two Day Old Pups Following Prenatal TCDD Exposure.

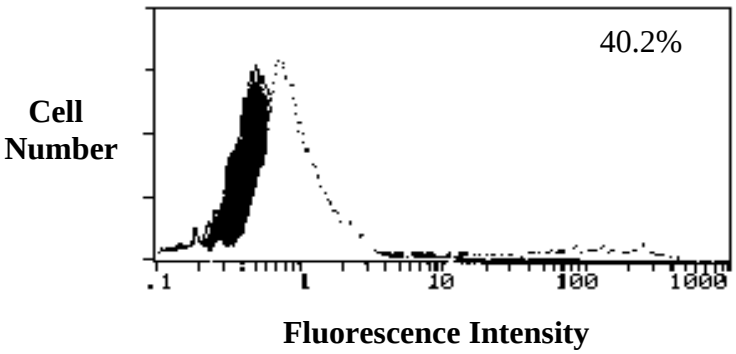


Figure 2. Detection of apoptosis *in vivo* in 2 day old pups. Pregnant mice were treated with various doses of TCDD or the vehicle control via a single intraperitoneal injection on gestational day 14. Thymectomies were performed on pups on postnatal day 2. Freshly isolated thymocytes were stained with TdT + FITC-dUTP and measured for apoptosis on a flow cytometer. TCDD treatment is represented by the white histogram and the vehicle control is represented by the dark histogram. Data shown are a representative experiment using a 1 μg/kg body weight dose of TCDD. A 40.2% increase in apoptosis was detected in thymocytes from the TCDD treated neonate (n=1) when compared to the vehicle control (n=1).

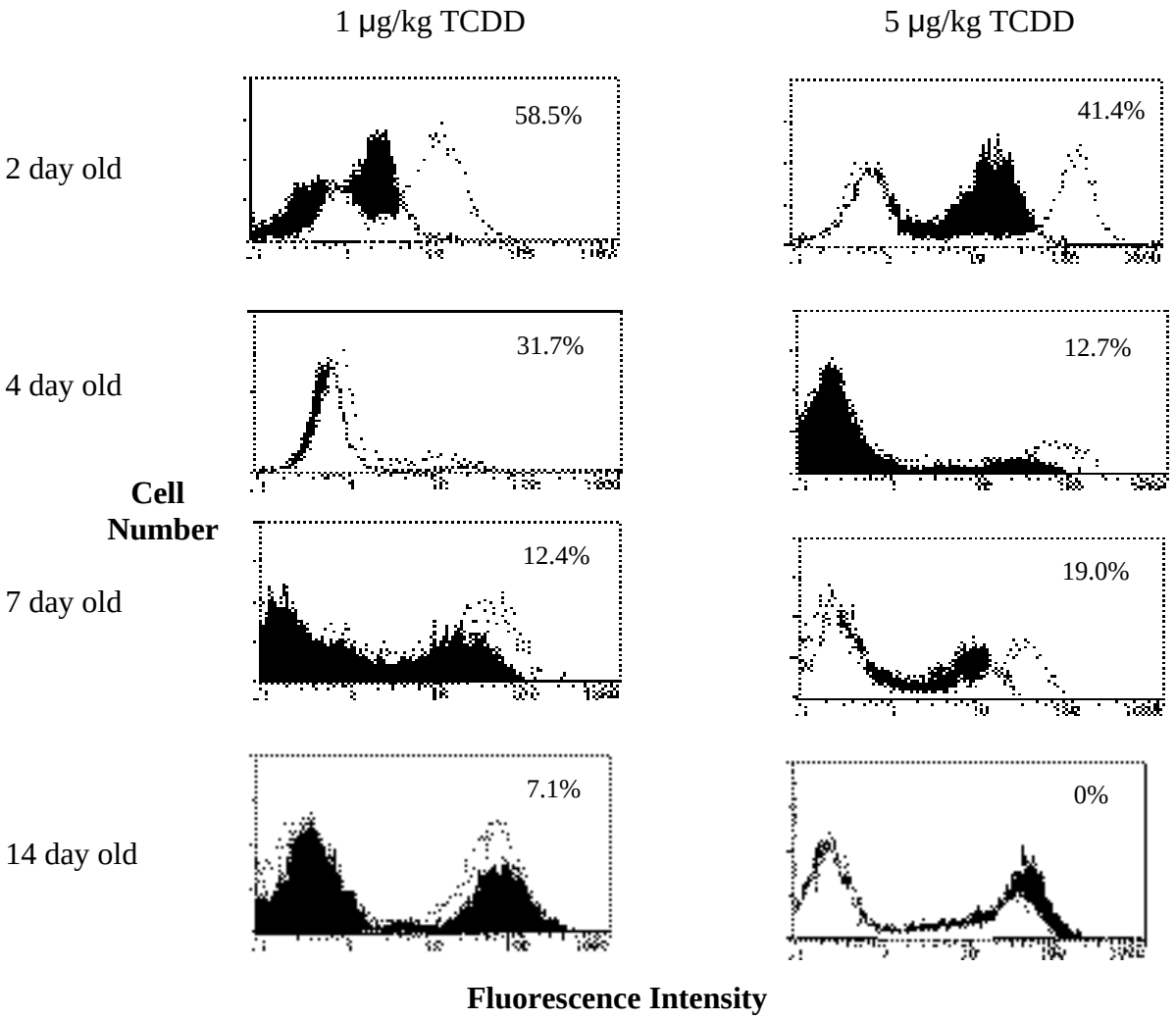


Figure 3. Detection of apoptosis in neonatal thymocytes after 24 hour *in vitro* culture. Pregnant mice were treated with various doses of TCDD or the vehicle control via a single intraperitoneal injection on gestational day 14. Thymectomies were performed on pups on postnatal days 2, 4, 7, and 14. $1x10^6$ thymocytes per well were cultured in a 96-well culture plate in a total of 0.2 mls of medium for 24 hours. The thymocytes were next harvested and stained with TdT + FITC-dUTP and measured for apoptosis on a flow cytometer. TCDD treatment is represented by the white histogram and the vehicle control is represented by the dark histogram. The dark histogram was subtracted from the white histogram to obtain the percentage of cells showing increased fluorescence, which is depicted in each panel.

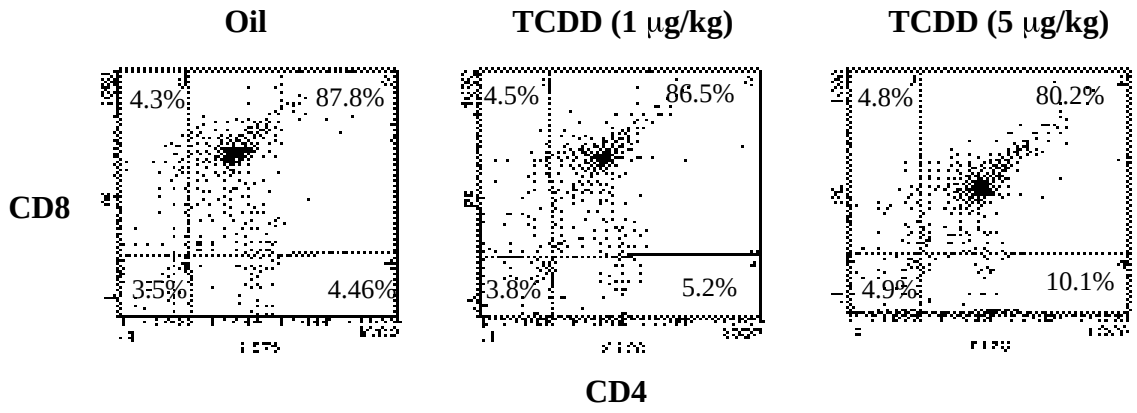


Figure 4. Effect of TCDD on T cell subsets of perinatally exposed mice. Pregnant mice were treated with various doses of TCDD or the vehicle control via a single intraperitoneal injection on gestational day 14. Thymectomies were performed on pups on postnatal day 2. Freshly isolated thymocytes were stained with FITC-CD4 and PE-CD8 antibodies. The cells were analyzed for double-staining using a flow cytometer. Data shown are representative of two experiments.

Previous studies suggested that there are changes in the T cell surface markers of adult mice following TCDD exposure (Kamath, et al., 1999). In 2 day old pups, we found that CD3, αβ−TCR, IL-2R, CD44, and J11d markers showed little change following exposure to 1 μg/kg TCDD (Figure 5). However, at 5 μg/kg dose of TCDD, the percentage of cells bearing CD3, αβ-TCR, IL-2R, and CD44 decreased markedly, while the mean fluorescence intensity did not alter significantly (Figure 5).

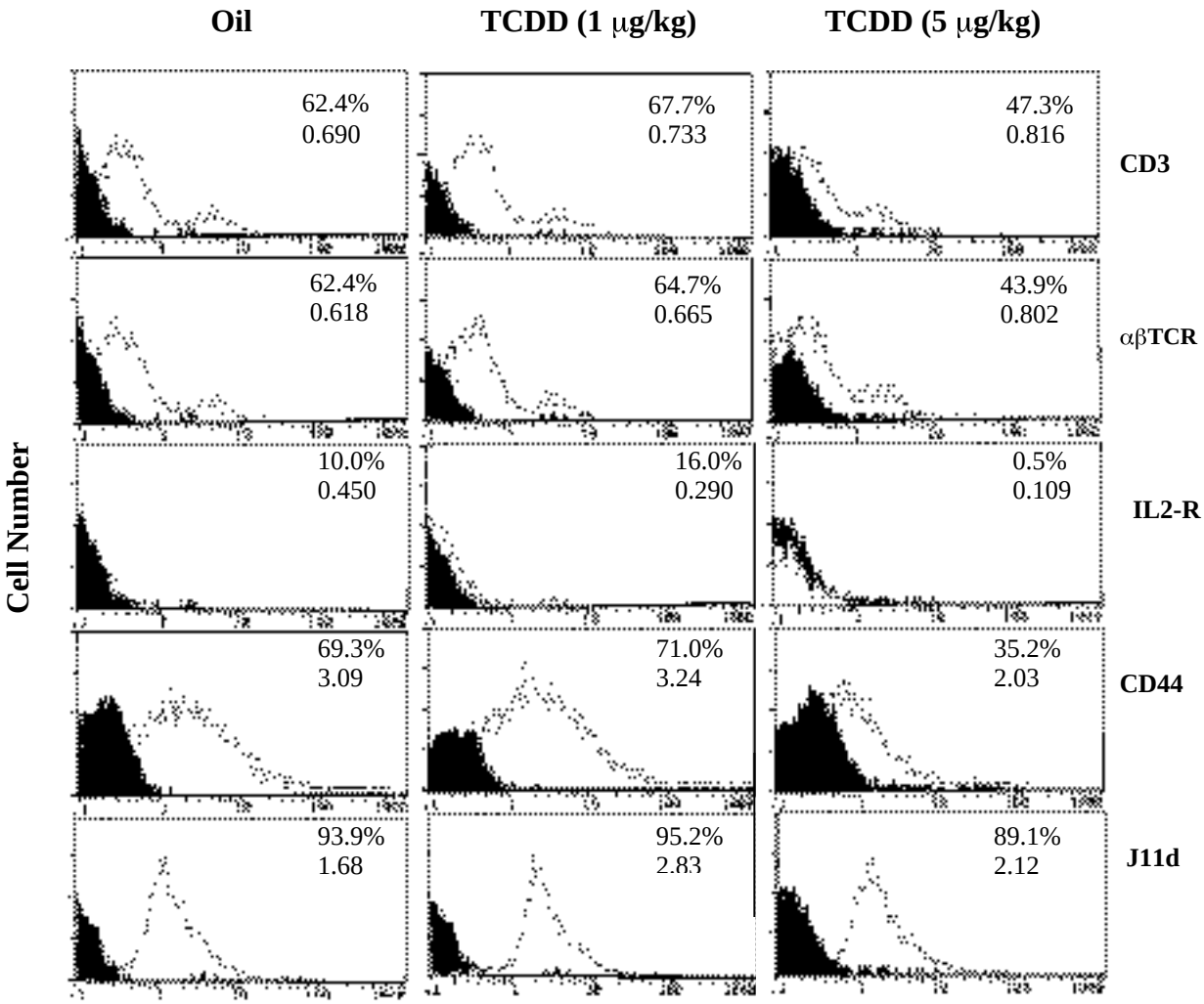


Figure 5. Phenotypic changes in thymocytes of 2 day old pups following perinatal TCDD exposure. Pregnant mice were treated with various doses of TCDD or the vehicle control via a single intraperitoneal injection on gestational day 14. Thymectomies were performed on pups on postnatal day 2. Freshly isolated thymocytes were stained with CD3, αβ-TCR, IL-2R, CD44, and J11d. The expression of cell-surface markers was measured by flow cytometry. The empty histograms represent cells stained with antibodies while the filled histograms represent cells stained with medium alone. The filled histogram was subtracted from the empty histogram to depict percentage of cells expressing various markers. Mean fluorescence intensity (MFI) is also shown. Data shown are representative of two experiments.

Discussion

In the current study, we found that perinatal exposure to TCDD led to decreased thymic cellularity in 14 and 21 day old pups, but not 2, 4, or 7 day old pups. Additionally, we detected apoptosis in 25% of 2 day old pups treated with TCDD in freshly isolated thymocytes. An increase in apoptosis was also detected in 2, 4, and 7 day old pups following 24-hour culture when compared to the vehicle-treated pups. Little or no increase in apoptosis was detected in the older 14 and 21 day old pups following 24-hour culture. However, an increase in apoptosis was detected in the 14 and 21 day old pups following 12 or 18 hour *in vitro* culture. We found that there was no change in the proportion of T cell subsets in the thymus of pups following perinatal exposure to TCDD when compared to the vehicle control. The percentage of T cells bearing markers such as CD3, αβ-TCR, IL2-R, CD44, and J11d was markedly decreased following exposure to the 5 μg/kg dose of TCDD.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, commonly known as "dioxin" or TCDD, is the most biologically potent of the halogenated aromatic hydrocarbons (HAH), and is considered to be the most toxic chemical ever created (Holsapple, 1991). It is an environmental contaminant formed as a byproduct in the manufacture of products from chlorinated phenols or during combustion of chlorinated materials. HAH have also been associated with herbicides, pulp and paper manufacturing, automobile exhaust from leaded gasoline, and combustion of municipal and industrial wastes (Holsapple, 1991). The immune system is highly sensitive to TCDD and therefore has raised significant health concerns, especially considering that HAH persist in the environment (Goldstein, 1989). TCDD causes decreased immunocompetence, even at doses that do not produce organ toxicity, and induces thymic involution as well as suppress T and B

lymphocyte functions (Luster, 1987, Rhile, 1997, Thomas, 1985, and Vos, 1980). The exact mechanism by which TCDD induces immunosuppression is unknown.

There are several proposed mechanisms for TCDD-induced thymic atrophy including damage to extrathymic T cell precursors in bone marrow and fetal liver (Silverstone, 1994) and initiation of apoptosis in the thymus (Kamath, 1997).

Kamath, et al. (1997) demonstrated that freshly isolated thymocytes from TCDD-treated adult mice failed to exhibit apoptosis upon direct testing. However, when such cells were cultured *in vitro* for 24 hours, enhanced apoptosis was detected in TCDD-exposed thymocytes. Similar observations were also made using dexamethasone, a well established inducer of apoptosis. In the current study, a similar observation was made except that in 25% of the experiments involving 2 day old pups exposed to TCDD, a significant increase in apoptosis was detected *in vivo* when compared to the control.

Apoptosis induced by chemicals is difficult to detect in freshly isolated thymocytes because of rapid clearance of apoptotic cells by the phagocytic cells found in the thymus (Savill, et al., 1993, Surh and Sprent, 1994). Thus, additional *in vitro* culture of thymocytes allows the detection of apoptotic cells due to the inability of phagocytic cells to clear the apoptotic cells rapidly. Previous studies show that in adult mice, alterations in the density of expression of cell surface markers can be used as a more reliable marker of apoptosis (Kamath, et al., 1999). Also, studies show that mice deficient in Fas or FasL are more resistant to immunotoxicity induced by TCDD (Kamath, et. al., 1998). Because Fas and FasL are linked to apoptosis, the above studies demonstrated that TCDD induces apoptosis and that this may play a key role in inducing immunotoxicity.

In the current study, we noted that TCDD induced marked decrease in thymic cellularity only in 14 and 21 day old pups but not in 2, 4, and 7 day old pups. We speculate that in the 2, 4, and 7 day old pups the cellularity is not significantly decreased following TCDD treatment due to the rapidly developing immune system of the younger pups. As the pup ages, the rate of growth slows, and therefore, the toxicity induced by the TCDD may become apparent leading to decreased thymic cellularity in the 14 and 21 day old pups. In addition, the pups are exposed to the TCDD through mother's milk, which may have a cumulative toxic effect in the pups. This may also explain why there was not a dose-dependent response, as the pups may receive different amounts of milk postnatally. Other studies report that a single-dose exposure of pregnant mice to TCDD in the microgram per kilogram range causes fetal thymic involution as well as inhibition of thymic differentiation, leading to inhibited T-cell responses lasting several weeks after birth (Holladay and Smialowicz, 2000). Studies show that TCDD reduces the capacity of fetal liver to synthesize TdT, which suggests an altered stem cell population in TCDD-treated mice that may be responsible for thymic atrophy (Holladay and Luster, 1995), since TdT^+ cells in fetal liver include prothyomcytes (Gregoire, et al., 1977).

Kamath, et al. (1998) reported that there are phenotypic changes in the adult C57BL/6 thymus following TCDD treatment, including upregulation in the density of expression of CD3, TCR, CD44, and IL-2R, and downregulation of CD4, CD8, and J11d. In contrast, the percentage of cells expressing these markers was not significantly altered. In the current study, however, the perinatally exposed pups did not show a significant change in the density of expression of various cell surface markers. Interestingly, at higher doses of TCDD, the thymocytes from the pups showed a marked decrease in the percentage of cells expressing CD3, αβTCR, IL-2R, CD44, and J11d. These data suggest that the thymocytes of adult mice and

young pups respond differently to TCDD. Previous studies also reported changes in the T cell subsets of pups prenatally-exposed to TCDD (Blaylock, et al., 1992). However, in the current study, we did not see such alterations, this may be because of the method of TCDD administration and the length of exposure differed in the studies.

In summary, the current study suggests that perinatal exposure to TCDD leads to induction of apoptosis and thymic atrophy. Because the thymus is crucial to the development of mature T cells, and apoptosis plays an important role in the selection of T cells, TCDD exposure could cause significant dysregulation in T-cell maturation and differentiation.

Chapter 4: Assessing Effects of Environmental Pollutants on *Bufo marinus* (Amphibia: Bufonidae) and Two Species of *Eleutherodactylus* (Amphibia: Leptodactylidae) in Bermuda*

L M Hudson Pélanne[1], M Nagarkatti[2], D Linzey[3], J B Linzey[4], J Robertson[2], C Brecht[3], J Burroughs[5], J Bacons[6], and P S Nagarkatti[1]. [1]Dept. of Biology, [2]Dept. of Biomedical Sciences, and [5]Dept. of Biochemistry, Virginia Tech, Blacksburg, VA, USA, [3]Wytheville Community College, Wytheville, VA, [4]New River Community College, Dublin, VA, and [6]Bermuda College, Paget, Bermuda.

*Major portions of this manuscript were provided by Dr. D. Linzey, the primary investigator in this study.

Abstract

Recently, a decrease in the populations of marine toads (*Bufo marinus*) and whistling frogs (*Eleutherodactylus johnstonei* and *E. gossei*) has been noted on the island of Bermuda. In the current study, we investigated whether this decline was related to altered immune functions. During August 1998, a significant proportion (25%) of the toads exhibited deformities. Analysis of soil samples revealed presence of chlorinated pesticides and polychlorinated biphenyls (PCBs). Spleen cells from toads collected from more polluted areas of Bermuda exhibited a decrease in B-cell proliferative response to lipopolysaccharide when compared to the responsiveness of B cells from toads collected from less polluted areas. In contrast, the T-cell responsiveness to mitogens in these two groups was not significantly altered. Histological examination of major parenchymatous organs in marine toads and whistling frogs showed alterations in hepatic and splenic morphology, indicative of exposure to toxicants. Together, the current study suggests that the immune functions of toads from contaminated sites of Bermuda are significantly altered, which may contribute to the decline in their population.

Introduction

Amphibians may signal environmental stress earlier than most other organisms and may serve as critical bio-indicators of ecosystem health. They may be sensitive to global changes of the environment on the planetary or continental scale, or they may be sensitive to local modifications of their environment. At least 16 countries on six continents have reported massive declines and die-offs among their amphibians since the 1970's. Species have apparently vanished from places as different as the Swiss Alps, Denmark, Nova Scotia, Peru, Panama, Puerto Rica, India, and the western United States. This disappearance of amphibians, particularly those in remote areas, suggests a general degradation of the environment, possibly worldwide in scope.

Bermuda is inhabited by three anurans: the marine toad (*Bufo marinus*) and two species of whistling frogs (*Eleutherodactylus johnstonei* and *E. gossei*). *Bufo marinus* and *Eleutherodactylus gossi* appear to have been declining in Bermuda since about 1987, according to interviews with local residents and with personnel of the Department of Agriculture, Fisheries, and Parks (Dow, 1993). *E. gossei* was last observed in July 1994 and may already be extirpated in Bermuda.

Numerous theories are proposed to explain declining amphibian populations. In some areas, an imbalance in small mammal populations that prey on amphibians, severe frosts, disappearing food sources, introduction of predators, human predation, and deforestation may be critical factors. In other areas, including Bermuda, major hypotheses include habitat acidification (Pierce, 1985; Freda and Dunson, 1986; Dunson, et al., 1992; Sadinski and Dunson, 1992; Warner, et al., 1993), heavy metal and pesticide contamination (Hall and Mulhern, 1984; Hall and Henry, 1992; Russell, et al., 1995), the effects of nitrate and nitrite from fertilizers

(Hecnar, 1995; Marco, et al., 1999); the presence of a particular fungus (Berger, et al., 1998; Kaiser, 1998), and immunological suppression (Carey, et al., 1999).

Bufo marinus breeds in fresh and/or brackish water. The eggs hatch into free-swimming aquatic larvae (tadpoles) before metamorphosing into the adult form. The two whistling frogs, however, reproduce by direct development. The eggs are deposited in moist terrestrial sites, and the entire aquatic stage takes place in the watery medium contained within the egg membrane. This prolongation of the egg stage significantly reduces mortality, although fewer eggs are produced because each embryo needs a large quantity of yolk for nourishment until hatching. Studies of rainwater in Bermuda have revealed that precipitation is acidic with a possible seasonal element. Rainfall data gathered by Jickells, et al. (1982) showed a mean pH of 4.74. Maximum acidity occurs during the winter.

Materials and Methods

Soil and water monitoring. A study of potential stressors was begun in March 1995, with primary emphasis being placed on gathering baseline data (Linzey, 1997). Two randomly-collected sets of soil samples were gathered from 15 permanent study sites three times each year. Two water samples were gathered from four study sites three times each year. One set of soil and water was delivered to the Soils Laboratory of the Bermuda Department of Agriculture, Fisheries, and Parks where all pH determinations were made. The other set was taken to Virginia for pesticide and heavy metal analyses. Pesticide analyses of water, soil, and tissues were performed in the Pesticide Laboratory of the Department of Biochemistry at Virginia Tech in Blacksburg, Virginia. Soil and tissue analyses for heavy metals were performed in the Chemistry Department at Virginia Tech. Water samples were analyzed for heavy metals by Olver Laboratories Incorporated in Blacksburg, Virginia. Five of the study sites were used for transect studies to monitor populations.

pH measurements. A technique was developed using a 1 cc syringe with a 28G (12 mm) micro-fine needle to successfully withdraw the intramembranous fluid from a whistling frog egg in order to determine the pH of the fluid bathing the developing embryo. The drop of fluid was placed on a strip of pH paper and the pH was recorded.

Parasite analysis. Specimens for parasite analyses were frozen as quickly as possible following capture. Later, each individual was thawed, measured, and weighed. The lungs, liver, gall bladder and bile duct, stomach, small intestine, large intestine, and urinary bladder were removed

and examined separately using dissecting and compound microscopes. Nematodes were identified using the standard glycerol wet-mount procedure. Trematodes were stained with Ehrlich's hematoxylin and mounted in Canada balsam. Selected helminths were deposited in the U.S. National Parasite Collection (USDA, Beltsville, Maryland) (Linzey, et al., 1998a, 1998b). All specimens for food analyses were frozen as quickly as possible following capture. Later, each individual was thawed, measured, and weighed on a Model S-400 Mettler balance. Contents of the stomach, small intestine, and large intestine were removed and examined separately under a binocular dissecting microscope. Frequency and volume of items contained in each stomach were recorded. Volume was determined by visual estimate. Animal prey were identified to the lowest possible taxon and quantified (Linzey, 1998c).

Pesticide analysis. The Bermuda Department of Agriculture, Fisheries, and Parks provided a list of 48 pesticides, herbicides, and fungicides registered for agricultural use in Bermuda. In addition to analyzing the tissues, water, and soil for these pesticides, any others that were identified during the analytical procedures were also recorded. All water and soil samples were stored at 4º C; all tissues and digestive system content samples were stored at -18º C. Tissue preparation for pesticide analyses involved weighing, measuring, and sexing the frog or toad and dissection of selected organs and/or tissues. The method chosen for extraction of pesticide residues from soil was an EPA method modified by the Virginia Tech Pesticide Research Laboratory (Watts, 1980). Extraction of pesticide residues from animal tissues was done by a method known as matrix solid-phase dispersion (MSPD). All determinations were made using gas chromatography with electron capture (GE-EC) detection.

Metal analysis. Heavy metal analyses were conducted for cadmium (Cd), chromium (Cr), copper (Cu), and zinc (Zn) in soil, water, and toad tissues collected in March and August 1998. Soil samples were analyzed by the methods of hot acid extraction and Inductively Coupled Plasma-Atomic Emission Spectrophotometry (ICP-AES). Each sample was tested three times for each metal. Water samples were analyzed using the Graphite Furnace Atomic Absorption Spectrometry (GFAA) method. The Varion Atomic Absorption Spectrometry procedure was used to analyze toad liver tissue.

Lymphocyte analysis. *Bufo marinus* and *E. johnstonae* were collected from polluted and less polluted sites in Bermuda. They were shipped to the lab site and allowed to acclimate for several days before the organs were harvested. Splenectomies were performed aseptically and the spleen placed in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10mM Hepes, 1mM glutamine, 40 μg/mL gentamicin sulfate, and 50 μM 2-mercaptoethanol. Single-cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation. The splenocytes were subjected to ficoll-hypaque density gradient centrifugation to isolate the lymphocyte population from the erythrocytes. Isolated lymphocytes were then further washed three times with RPMI medium.

Total cellularity. Lymphocytes prepared as described above were resuspended in medium. Twenty-five microliters of the single-cell suspension were added to 100 microliters of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under an inverted-phase contrast microscope.

Lymphocyte proliferation. Proliferation assay (Rhile, et al., 1996) was used to study the DNA synthesis in *B. marinus* lymphocytes from the polluted and less polluted areas. Varying cell concentrations from $6x10^5$ to $1.2x10^6$ cells per well in 0.2 mls of medium were stimulated with varying concentrations of mitogens, including phorbol ester (PMA) (10 ng/ml) and calcium ionophore (0.5 μM), phytohemmaglutinin (PHA) (50 μg/ml), concanalvalin A (ConA) (2 μg/ml), and lipopolysaccarhide (LPS) (50 μg/ml) (Sigma, St. Louis, MO). The assay was run in a 96-well tissue culture plate that was incubated at 37°C for 24-48 hours. Six to eighteen hours before harvesting, the plates received 50 μL of ^{3}H-thymidine. The cells were harvested using an automated cell-harvesting machine (Skatron, Sterling, VA). The radioactivity was analyzed in a liquid scintillation counter (Tm Analytical, Elk Grove Village, IL). Results were expressed as radioactive counts per minute (CPM). In addition, in all experiments stimulation index was calculated by dividing the CPM obtained in cultures receiving mitogens with CPM seen in medium controls. Data showing a stimulation index of 2 or greater were considered for analysis.

Histology. *Bufo marinus* and *E. johnstonae* were collected from polluted and less polluted sites in Bermuda. They were shipped to the lab site and allowed to settle for several days before the organs were harvested. Liver, lung, spleen, ovary, testes, and thymus were fixed in 10% formalin after harvesting and studied histologically using hemotoxylin and eosin stain.

Statistical Analysis. Statistical analysis was performed by the Statistical Consulting Center of Virginia Tech. Student's t test was used to compare the proliferative results. $P<0.05$ was considered to be significant.

Results

Between July 1995 and March 1999, a total of 38 *Eleutherodactylus* egg masses were located, nine of which were guarded by an adult frog. The number of eggs per mass ranged from 1 to 82 (mean =24.1). The most common sites for egg deposition were on the ground in soft palmetto tissue and inside or beneath banana logs. Six egg masses disappeared several days following their discovery. In addition, two egg masses were invaded by fungi, one of which was cultured and identified as *Pythium* species. Ants were observed on one egg mass.

The soil pH of the 15 study sites ranged from 4.8 to 8.8. The pH of the water at four sites ranged from 6.7 to 8.6. Between July 1995 and March 1999, a total of 19 *Eleutherodactylus* egg pH determinations were recorded. They ranged from 4.6 to 7.4 (mean=pH 6.0).

Three nematode species (*Rhabdias fuelleborni, Aplectana* sp., and *Abbreviata* sp.) and two trematode species (*Mesocoelium monas* and *Clinostomum* sp.) were found in *Bufo* (Linzey, et al., 1998b). The overall infection rate was 93%. Parasite loads were high: rates of infection for *Rhabdias* ranged from 25% to 100%; for *Mesocoelium*, they ranged from 0% at two study sites to 100%. One individual contained 114 lung nematodes (*Rhabdias*), while another harbored over 2000 trematodes (*Mesocoelium*).

Of even greater significance is the apparent dramatic increase in trematode infection in *Bufo* at one site. Goldberg (1995a) reported the trematode *Mesocoelium* in only one out of 45 toads (2%) taken from the Bermuda Biological Station in August 1992. The first collection of toads from this site during the current study was in March 1995 when 14 out of 26 toads (53.8%) were found to be infected with this trematode. The prevalence of *Mesocoelium* at this site has never been below 45% (July 1995- 45%; November 1995 -63%; August 1996- 95%; May 1997-

90%; August 1998- 90%; March 1999- 90%). Parasite identifications were made by the same parasitologist in both studies. The situation at this site in Bermuda will continue to be monitored.

Four species of nematodes (*Parapharyngodon garciae, Aplectana* sp., *Abbreviata* sp., and *Batracholandros* sp.) and two species of trematodes (*Mesocoelium monas* and an unidentified species belonging to the family Opecoelidae) were identified in *E. johnstonei* (Linzey, et al., 1998a). Rates of infection ranged from 67% (1 site) to 100% (4 sites). Parasite loads varied seasonally (*Parapharyngodon* - 19% to 44%; *Aplectana* - 31% to 36%; and *Abbreviata* - 64% to 74%). Two helminths (*Batracholandros* sp. and the opecoelid trematodes) represent new records of parasitism for *E. johnstonei* and for Bermuda.

Major spring and summer food items of 203 *Eleutherodactylus johnstonei* in descending order of percentage volume were millipedes, sowbugs, ants, beetles, insect larvae, and spiders (Linzey, et al., 1998c). Major spring and summer food items of 80 *Bufo marinus* in descending order of percentage volume were vegetation, millipedes, beetles, insect larvae, and ants (Linzey, et al., 1998c). Cockroaches were absent from toad stomachs during March but comprised as much as 46% of the total volume of food during July.

No pesticide residues were found in any water sample. However, in the soil samples and in frog and toad tissues the most abundant chemical pesticide residue was p,pDDE, a breakdown product of either DDT or Kelthane, both of which have been used in Bermuda and most other countries of the world. DDT was banned in Bermuda in the early 1970s; Kelthane is still used. DDE was found in the soil from every study site in concentrations ranging from 0.003 to 4.023 ppm. DDE was found in concentrations ranging from 0.027 to 1.626 ppm in toad liver tissue at five of the study sites where toads were present. The fat bodies from toads from four sites

yielded p,pDDE concentrations of 0.192, 0.551, 2.277, and 12.170 ppm. DDE was found in whistling frogs from six out of six sites at concentrations ranging from 0.054 to 1.145 ppm. Preliminary analyses of earthworms, millipedes, cockroaches, and sowbugs from one study site revealed DDE concentrations ranging from 0.050 to 0.217 ppm. For comparative purposes, three soil samples and invertebrates including ants, beetles, centipedes, grubs, sowbugs, and earthworms were obtained from a study plot on the Virginia Tech campus by a member of the Pesticide Laboratory. The soil and invertebrates were analyzed in the same manner and by the same personnel as the soil and invertebrates from Bermuda. The three soil samples yielded p,pDDE values of 0.000 ppm, 0.001 ppm, and 0.026 ppm. Every invertebrate sample had a p,pDDE level of 0.000 ppm.

Other pesticides found in preliminary analyses of Bermuda soil included DDT at 8 sites, Kelthane at 8 sites, and dieldrin at 3 sites. Although polychlorinated biphenyls (PCBs) are not pesticides, these compounds are frequently analyzed along with pesticides. PCBs were identified at 9 of the study sites.

The muscle and skin of some *Eleutherodactylus* and *Bufo* were analyzed for heavy metals at the Bermuda Biological Station for Research in 1997. Analyses were carried out for three metals - cadmium, chromium, and lead. Cadmium was found in whistling frogs from 11 of 12 study sites at concentrations ranging from 0.01 to 14.20 micrograms per gram of tissue; chromium was found in whistling frogs from 12 out of 12 study sites at concentrations ranging from 0.28 to 12.79 micrograms per gram of tissue; and lead was found in whistling frogs from 12 out of 12 study sites at concentrations ranging from 0.01 to 11.30 micrograms per gram of tissue. Analyses of *Bufo* tissues revealed cadmium in toads from 2 of 6 study sites at a concentration of 0.01 micrograms per gram of tissue; chromium in toads from 6 of 6 study sites at concentrations

ranging from 0.62 to 6.90 micrograms per gram of tissue; and lead in toads from 6 of 6 study sites at concentrations ranging from 0.02 to 6.44 micrograms per gram of tissue. Analyses of March and August 1998 water samples from 5 study sites showed low levels of cadmium (0.0001 to 0.0010 mg/L), low levels of chromium (non-detectable to 0.003 mg/L), relatively low levels of copper (0.001 to 0.049 mg/L), and low levels of zinc (non-detectable to 0.009 mg/L). Analyses of March and August 1998 soil samples from 9 study sites revealed the presence of cadmium at 8 sites (0.4 to 22.7 ppm), chromium at 9 sites (1.6 to 529.5 ppm), copper at 8 sites (11.0 to 224.6 ppm), and zinc at 9 sites (5.8 to 449.0 ppm). Analyses of livers from 14 *Bufo marinus* collected in March and August, 1998 from two study sites revealed the presence of cadmium, chromium, copper, and zinc in all 14 specimens at the following concentrations: cadmium (0.03 to .275 ppm); chromium (0.04 to 1.20 ppm); copper (12.7 to 803.33 ppm); and zinc (12.07 to 31.00 ppm).

Examination of organs (liver, spleen, and thymus) from two *Bufo marinus* from the same study site in Bermuda showed significant alterations in hepatic and splenic morphology when compared to *Bufo marinus* from Mexico. Livers of Bermuda specimens exhibited decreased hepatocytes and an increased number of pigmented macrophages (melanomacrophages), which had become enlarged and very prominent; these conditions were not present in the Mexican control specimen (Figure 1). Livers from Bermuda also contained spindle-cell inclusions and possible granulomas. Spleens of Bermuda toads showed a marked decrease in white pulp. The livers and spleens of three *Eleutherodactylus johnstonei* from Bermuda showed extreme variation between study sites. The liver of one frog showed a great amount of clear cell vacuolization with an abundance of glycogen, whereas the liver of a whistling frog from a second study site showed a significant decrease in hepatocytes. Many intercellular spaces were

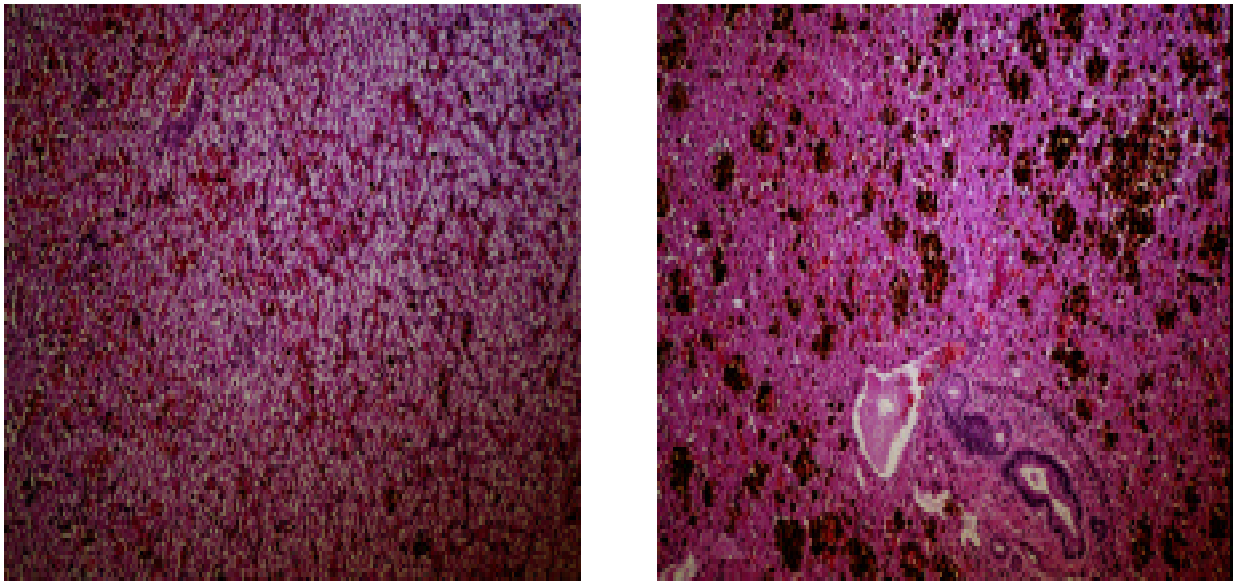


Figure 1. Livers of *B. marinus* collected in Bermuda exhibit histological differences when compared to control toads from Mexico. The panel on the left depicts a liver from a control *B. marinus* specimen, while the panel on the right depicts a representative example of a *B. marinus* liver from a specimen collected in Bermuda. Note the grossly increased number of melanomacrophages in the Bermuda specimen. Tissue sections were stained with hematoxylin and eosin stain.

present in the livers and spleens of both *Bufo marinus* and *Eleutherodactylus johnstonei* from Bermuda.

Splenocytes from *Bufo marinus* were harvested on three different occasions and tested for proliferative responses *in vitro*. The toads were collected from BBSR, considered to be more polluted, and the Zoo site, considered to be less polluted. The T lymphocytes were stimulated using various mitogens including Concanalvalin A (ConA) and phytohemmaglutinin (PHA). The B lymphocytes were stimulated using lipopolysaccharide (LPS). We found a significant decrease in the proliferative response of toads from BBSR to LPS when compared to the Zoo site (Figure 2). There was no significant difference in proliferative ability to PHA or ConA. These data suggest that the levels of pollution found at the BBSR site may be affecting the ability of B lymphocytes to proliferate, and therefore contribute to immunosuppression in the toads.

Over 600 *Bufo marinus* were handled in Bermuda between March 1995 and October 1999. Prior to August 1998, only one toad with an abnormally developed limb had been found. During August 1998, a total of 12 adult toads were examined from three study sites. Three toads (25%) from two of the study sites exhibited deformities. During 1999, between 15% and 25% of all toads examined from a variety of sites exhibited deformities in either the forelimbs or hind limbs. During October 1999, 21% of 52 toads at a study site where pesticides are not used had deformed toes.

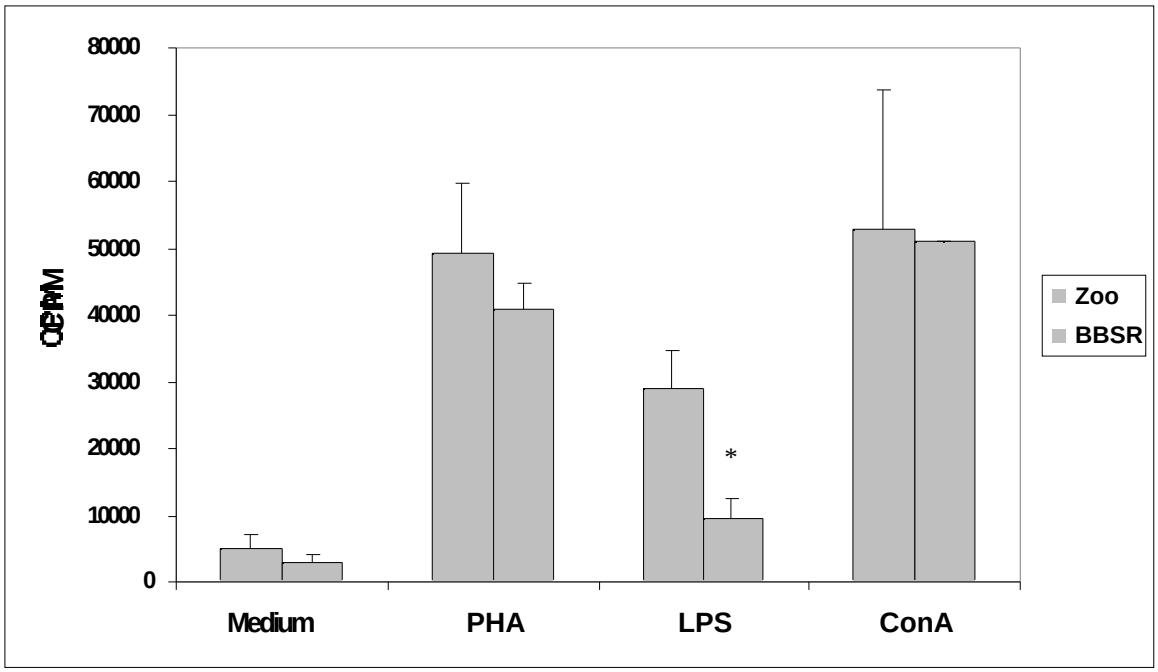


Figure 2. Proliferative responsiveness of *B. marinus* lymphocytes to mitogens. *B. marinus* were sampled from Zoo site (n=2) and from Bermuda Biological Station for Research (BBSR) (n=3). The spleens were harvested, lymphocytes purified, and cultured *in vitro* with 50 µg/ml PHA, 50 µg/ml LPS, and 2 µg/ml ConA for 24-48 hours at 37º C. Data represent mean +/- S.E.M. counts per minute derived from triplicate cultures. Asterix indicates statistically significant difference when compared to Zoo group ($p<0.05$).

Discussion

The present nematode and trematode infections of amphibians are significantly higher than those reported previously in Bermuda (Goldberg, et al., 1995a, 1995b). Infection rates to date are also higher than in these species of amphibians reported in the literature from elsewhere in the world (Lees, 1962; Baker, 1979; Goldberg and Bursey, 1992; and others). Johnson, et al. (1999) indicated a positive correlation between trematode infection and limb abnormalities. Johnson, et al. (1999) induced limb abnormalities at high frequencies in Pacific treefrogs (*Hyla regilla*) when they were exposed to cercariae of a trematode parasite (*Ribeiroia* sp.).

Growing concern about the decline of certain amphibian populations has led to renewed awareness of problems from pesticides. Testing of chemicals for chemical registration is done for mammals, birds, fish, and insects but not for amphibians. Research suggests that amphibians are more resistant to some chemicals, but more sensitive to others than could have been predicted by current testing procedures on fishes, birds, and mammals (Hall and Henry, 1992). Existing studies on amphibians usually are laboratory studies involving tadpoles only.

Organochlorine chemicals are known to pose a serious threat to certain insectivorous bat populations and may have played a role in past declines of many other animal populations (Hoffman, et al., 1990). Bioaccumulation of contaminants in food chains with resulting biomagnification in wildlife first became evident when mortality of American robins was linked to DDT in earthworms (Carson, 1962). Larval amphibians have exhibited an ability to store organochlorine insecticides in their fatty tissues, most importantly the tail. During resorption of the tail at metamorphosis, these residues are mobilized and lead to mortality among young frogs. Russell, et al. (1995) reported significant tissue accumulations of DDT, DDE, DDD, and dieldrin

in spring peepers (*Pseudacris crucifer)* 26 years after pesticide application was abandoned in southern Ontario. Thus, the hazard presented by some organochlorines may affect all stages of the amphibian life cycle long after their application.

Carbamate and organophosphate insecticides act by inhibiting acetylcholinesterase enzymes that are essential for nerve impulse conduction and transmission. Carbamates are moderately toxic to amphibians. Organophosphates, however, present the widest range of toxicity. Although numerous laboratory studies have been reported (Power, et al., 1989), few rigorous efforts have been made to investigate the effects of such chemicals on natural anuran populations. In laboratory studies, residue levels of organophosphates in *Rana catesbeiana* tadpoles showed significant bioaccumulation (Hall and Kolbe, 1980; Hall, 1990). Parathion was magnified an average of 64 times and fenthion 62 times. Maximum levels found in pooled samples were 196 ppm parathion and 320 ppm fenthion. However, mortality of ranid tadpoles did not begin until levels of parathion in the water approached 5 mg/L.

The ubiquitous presence of p,pDDE in the soil, in the invertebrates, and in the tissues of *Bufo marinus* and *Eleutherodactylus johnstonei* in Bermuda is alarming. Researchers at Odense University's School of Medicine in Denmark reported that both DDE and PCB levels were twice as high in humans who died from cancer as in those individuals who died of other illnesses (Jensen and Nordberg, 1980). Blood concentrations of two pesticides were reported as independent risks for breast cancer in a study of Danish women (Hoyer, et al., 1998). Blood concentrations of beta-hexachlorocyclohexane, a constituent of the toxic pesticide lindane, caused a woman's chance of developing breast cancer to increase slightly. The persistent insecticide dieldrin provoked a more dramatic increase. Women with the highest blood concentration of this estrogen-mimicking pollutant faced more than double the breast cancer risk

of those whose blood carried little or no dieldrin. As part of the present study, efforts are underway to determine the source of these chemicals in Bermuda. Whether the source is local or whether these chemicals are long-range contaminants from emissions in North America is unknown at present. Jickells, et al. (1982) stated that major weather patterns in the North Atlantic move in an easterly direction, thus transporting air masses from the United States towards Bermuda. However, data on the effects of wind-borne pollutants on amphibian populations are non-existent.

Simmons (1997) reported soil analyses of heavy metals from 11 sites in Bermuda. His results indicated lower levels of cadmium, chromium, copper, and zinc than were found in the March and August 1998 samples analyzed in this study. Direct comparisons were not possible because different sites were sampled in the two research efforts. Friberg, et al. (1986) stated that cadmium levels in soil are usually less than 1.0 ppm. The low excretion rates of cadmium lead to a very efficient retention of cadmium in the body. The half-life in the mouse and rat is 200-700 days; in squirrel monkeys, it is longer than two years; and in humans it may be 10-30 years (Friberg, et al., 1986).

Birge, et al. (1979) reported that after seven days, half of the embryo-larval stage of *Gastrophryne carolinensis* died when exposed to 0.04 mg/L of copper. Copper concentrations exceeded this level at one Bermuda study site in August 1998. This may have contributed to an increase in the mortality of the tadpoles and, therefore, a decrease in the adult population at this site. The average concentration of copper in the livers of *Bufo marinus* at one of the Bermuda study sites was 430.33 ppm, nearly four times the level of copper reported by Pasanen and Koskela (1974) in the livers of healthy *Rana temporaria*. Pasanen and Koskela (1974) reported an average zinc concentration of 18.0 ppm in *Rana temporaria*, while Byrne, et al. (1975)

recorded levels of zinc ranging form 11.0 to 28.3 ppm in the livers of healthy *Bufo trituris*. Hall and Mulhern (1984) cited cadmium levels in two *Bufo* spp. as ranging from 80 to 130 ppb. Seven of eight *Bufo marinus* from one of the Bermuda study sites contained cadmium levels above 130 ppb. Copper, lead and zinc are among the known contaminants from the East Coast of the United States. Knap and Kaiser (1990) calculated an estimate of the quantity of each element exported off the East Coast and the estimated deposition of each element west of Bermuda.

The abnormalities noted in the hepatocytes in the livers of *Bufo marinus* and *Eleutherodactylus johnstonei* could have been caused by a lack of food or by an enzyme deficiency. The many intercellular spaces in the livers and spleens are indicative of a decrease in leukocytes, the body's main line of defense against disease-causing organisms and a possible indication of immune system suppression. In addition, the decreased proliferative response of the toads collected in the more polluted BBSR site when stimulated with LPS suggests that the pollutants detected may contribute to suppression of the humoral branch of the immune system, either by suppressing the activity of the B-cells, or by decreasing the number of B cells available to mount an immune response. It is possible that a decrease in humoral immune-mediated response could account for increased incidence of parasitic infections.

The fact that 21% of 52 toads had deformed toes at a study site in October 1999 where pesticides are not used may be highly significant. Reportedly, a considerable amount of fertilizer is used at this site. Hecnar (1995) reported acute and chronic toxicity to tadpoles of four species of anurans (*Bufo americanus, Pseudacris triseriata, Rana pipiens,* and *Rana clamitans*) from ammonium nitrate fertilizer. Mortality varied among species and significant weight loss occurred.

When nitrate or nitrite ions were added to the water, some larvae of five species of pond-breeding amphibians showed reduced feeding activity, swam less vigorously, showed disequilibrium and paralysis, suffered abnormalities and edemas, and eventually died (Marco, et al., 1999). One aspect of future research efforts in Bermuda will be to quantify fertilizer use on all study sites and analyze the soil, water, and tissues for nitrate and nitrite.

The data presented in this paper on parasites, food analyses, distribution, fecundity, pH analyses, heavy metal and pesticide analyses, and preliminary immunological and pathological examinations of amphibian tissues now form the most complete and comprehensive database ever assembled for these species in Bermuda. These data provide the basis for future work on egg predation, habitat acidification, immunological competence, pathological studies, and additional pesticide and heavy metal analyses.

Chapter 5: Conclusions

In the current study, we addressed the effects of environmental pollutants on the immune system using fish, amphibian, and mammalian models. We found that certain fish can be used in the laboratory as models for immunosuppression induced by environmental contaminants and therapeutic drugs. Field studies involving fish were found to be more difficult than laboratory investigation due to variation of the habitat, temperature, and species. Overall trends of immunomodulation, however, can be studied using field techniques modified to allow for differences in these variables. Because they are frequently at the top of the food chain, fish may serve as an ideal forum to study the effect of aquatic contaminants on the immune system as well as act as a sensor for detection of hazards for human health.

In addition, we tested the hypothesis that perinatal exposure to TCDD, a highly toxic environmental contaminant, leads to immunotoxicity due to induction of apoptosis in thymocytes. We found a decrease in thymic cellularity in 2 and 3 week old pups following perinatal exposure to TCDD. The percent viability of thymocytes of pups exposed to TCDD was decreased when compared to the vehicle control. Using the TUNEL method, a percent increase in apoptosis was detected in 25% of freshly isolated thymocytes of 2 day old pups following TCDD treatment when compared to the controls. In addition, a percent increase in apoptosis was detected in the 2, 4, 7, 14, and 21 day old TCDD-treated pups after *in vitro* culture when compared to the vehicle controls. Following the 5 μg/kg dose of TCDD, a downregulation of phenotypic markers was detected on the thymocytes of 2 day old pups. These data support the hypothesis that apoptosis plays an important role in the toxicity induced by TCDD in perinatally-exposed pups.

We also investigated the immune response and histological changes in amphibians from polluted and less-polluted areas in Bermuda. We found that the toads collected from the polluted BBSR site had a significantly decreased proliferative response when stimulated with LPS when compared to the toads from the less polluted Zoo site, suggesting immunosuppression of the humoral branch of the immune system. We also found histological changes in the amphibians from Bermuda when compared to control amphibians from Mexico. Bermuda amphibians exhibited an increase in melanomacrophage centers in the liver and spleen, as well as a decrease in white pulp in the spleen, suggesting possible immunosuppression.

These data taken together encompass a broad study on the effect of environmental pollutants across species. In each study, immunosuppression is the end result of contact with contamination, whether occurring in the environment or induced in the laboratory. These data suggest that fish and amphibians should be considered as viable alternatives for immunotoxicity testing, as they frequently come into direct contact with environmental pollutants before mammalian species do, and may serve as biomarkers for human health hazards.

Literature Cited

Al-Bayati, Z.A., Murray, W., Pankaskie, M.C., Stohs, S.J. (1988). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induced perturbation of calcium distribution in the rat. *Res. Commun. Chem. Pathol. Pharmacol*. 60(1): 47-56.

Baker, M.R. (1979). Seasonal population changes in *Rhabdias ranae* Walton, 1929 (Nematoda: Rhabdiasidae) in *Rana sylvatica* of Ontario. *Canadian Journal of Zoology* 57: 19-183.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli, and H. Parkes. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Science, USA* 95: 9031-9036.

Birge, W. J., J. A. Black, and A. G. Westerman. (1979). Evaluation of aquatic pollutants using fish and amphibian eggs as bioassay organisms. *Animals as Monitors of Environmental Pollution*, pp. 108-118. Washington, D. C.: National Academy of Science.

Birnbaum, L.S., Morrissey, R.E., Harris, M.W. (1991). Teratogenic effects of 2,3,7,8-tetrabromodibenzo-*p*-dioxin and three polybrominated dibenzofurans in C57BL/6N mice. *Toxicol. Appl. Pharmacol.* 107(1): 141-152.

Blaylock, B L, Holladay S. D., Comment C. E., Heindel J. J., Luster M. I. (1992). Exposure to TCDD alters fetal thymocyte maturation. *Tox. Appl. Tox.* 112: 207-213.

Burkitt, H. G., Young, B., Heath, J.W. (1993). *Wheater's Functional Histology*. Churchill Livingstone, London, UK.

Byrne, A. R., L. Kosta, and P. Stegnar. (1975). The occurrence of mercury in amphibia. *Environmental Letters* 8: 147-155.

Carey, C., N. Cohen, and L. Rollins-Smith. (1999). Amphibian declines: an Immunological perspective. *Developmental and Comparative Immunology* 23: 459-472.

Carson, R. (1962). *Silent Spring*. Houghton Mifflin. Boston, Massachusetts.

Choi, E. J., Toscano D. J., Ryan J. A., Reidel, N., and Toscano, W. A. (1991). Dioxin induces transforming growth factor-alpha in human keratinocytes. *J. Biol. Chem.* 266: 9591-9597.

Clary, S., P. S. Nagarkatti, M. Nagarkatti. (1990). Immunomodulatory effects of nitrosoureas on the phenotype and functions of T cells in the thymus and periphery. *Immunopharmacology* 20(3): 153-64.

Collazos, M. E., Ortega, E., and Barriga, C. (1995). Influence of the Temperature upon the proliferative response of lymphocytes of Tench (*Tinca tinca*) during winter and summer. *Comp. Immun. Microbiol. Infect. Dis.* 18(3): 209-214.

Dickson, K. L. (1979). Aquatic ecological classification and evaluation of streams and stream reaches in the upper Roanoke River basin, Virginia. Wilmington District Corps of Engineers.

Dow, R. (1993). Island's toads and frogs dying out, says scientist. *Royal Gazette*. May 10, 1993:3.

Dunier, M. and A. K. Siwicki (1994). Effects of lindane exposure on rainbow trout (*Oncorhynchus mykiss*) immunity. I. Effect of lindane on antibody-secreting cells (ASC) measured by ELISPOT assay. *Ecotoxicol. Environ. Saf.* 27: 1-6.

Dunson, W. A., R. L. Wyman, and E. S. Corbett. (1992). A symposium on amphibian declines and habitat acidification. *Journal of Herpetology* 26(4): 349-352.

Faisal, M., Marzouk, M.S.M., Smith, C.L., Huggett, R.J. (1991). Mitogen Induced Proliferative Responses of Lymphocytes of Spot (*Leiostomus xanthurus*) Exposed to Polycyclic Aromatic Hydrocarbon Contaminated Environments. *Immunopharmacology and Immunotoxicology* 13: 311-327.

Fawthrop, D. J., A. R. Boobis, D. S. Davies. (1991). Mechanisms of cell death. *Arch Toxicol.* 65: 437-444.

Fine, J. S., A. E. Silverstone, T. A. Gasiewicz. (1990). Impairment of prothymocyte activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Immunol* 144(4): 1169-76.

Fine, J. S., T. A. Gasiewicz, N. C. Fiore, A. E. Silverstone. (1990). Prothymocyte activity is reduced by perinatal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure. *J Pharmacol Exp Ther* 255(1): 128-32.

Fine, J. S., T. A. Gasiewicz, A. E. Silverstone. (1989). Lymphocyte stem cell alterations following perinatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol Pharmacol* 35(1): 18-25.

Freda, J. and W. A. Dunson. (1986). Effects of low pH and other chemical variables on the local distribution of amphibians. *Copeia* 1986(2): 454-466.

Friberg, L., T. Kjellstrom, and G. F. Nordberg. (1986). Cadmium. *Handbook on the Toxicology of Metals. Second Edition. Volume II: Specific Metals*, pp. 130-184. L. Friberg, G. F. Nordberg, and V. B. Vouk, eds. Elsevier Publishing Company. Amsterdam.

Gehrs, B.C., Smialowicz, R.J. (1997). Alterations in the developing immune system of the F344 rat after perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin I. [correction of II]. Effects on the fetus and the neonate. Toxicology 122(3): 219-228.

Gregoire, K. E., Goldschneider, I., Barton, R. W., and Bollum, F. J. (1977). Intracellular distribution of terminal deoxynucleotidyl transferase in rat bone marrow and thymus. Proc Natl Acad Sci USA 74: 3993-3996.

Goldberg, S. R. and C. R. Bursey. (1992). Helminths of the marine toad, *Bufo marinus* (Anura: Bufonidae) from American Samoa. *Journal of the Helminthological Society of Washington* 59: 131-133.

Goldberg, S. R., C. R. Bursey, and R. Tawil. (1995a). Helminths of an introduced population of the giant toad, *Bufo marinus* (Anura: Bufonidae), from Bermuda. *Journal of the Helminthological Society of Washington* 62: 64-67.

Goldberg, S. R., C. R. Bursey, and R. Tawil. (1995b). Gastrointestinal helminths of *Eleutherodactylus johnstonei* (Leptodactylidae) from Bermuda. *Journal of the Helminthological Society of Washington* 62: 67-69.

Goldstein, J.A., et.al. (1989). Mechanism of action and structure-activity relationship for the chlorinated dibenzo-*p*-dioxins and related compounds. *Halogenated Biphenyls, Terphenyls, Naphalenes, Dibenzodioxins, and Related Products*, ed. R. D. Kimbrough, A. A. Jensen, 4: 239-93. Amsterdam: Elsevier.

Graves, S. S., Evans, D. L., Cobb, D., Dawe, D. L. (1984). Nonspecific Cytotoxic Cells in Fish (*Ictalurus punctatus*) I. Optimum Requirements for Target Cell Lysis. *Developmental and Comparative Immunology* 8: 293-302.

Hall, R. J. (1990). Accumulation, metabolism and toxicity of parathion in tadpoles. *Bulletin of Environmental Contamination and Toxicology* 44: 629-635.

Hall, R. J. and P. F. P. Henry. (1992). Assessing effects of pesticides on amphibians and reptiles: status and needs. *Herpetological Journal* 2: 65-71.

Hall, R. J. and E. Kolbe. (1980). Bioconcentration of organophosphorus pesticides to hazardous levels by amphibians. *Journal of Toxicology and Environmental Health* 6: 853-860.

Hall, R. and B. Mulhern. (1984). Are anuran amphibians heavy metal accumulators? *Vertebrate Ecology and Systematics*: 123-133.

Heath, Alan G, (1995). *Water Pollution and Fish Physiology*. CRC Press, Inc., Boca Raton, Florida.

Hecnar, S. J. (1995). Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environmental Toxicology and Chemistry* 14(12): 2131-2137.

Hinton, D. and Laurén, D. J. (1990). Integrative Histopathological Approaches to Detecting Effects of Environmental Stressors on Fishes, *American Fisheries Society Symposium* 8: 51-66, in Biological Indicators of Stress in Fish, Adams, S. M., Ed.

Hoffman, D. J., B. A. Rattner, and R. J. Hall. (1990). Wildlife toxicology. *Environmental Science and Technology* 24(3): 276-283.

Holladay, S. and Luster, M. (1995). Alterations in Fetal Thymic and Liver Hematopoeitic Cells as Indicators of Exposure to Developmental Immunotoxicants. *Environmental Health Perspectives* 104(4), 809-813.

Holladay, S. and Smialowicz, R. (2000). Development of the Murine and Human Immune System: Differential Effects of Immunotoxicants Depend on Time of Exposure. *Environmental Health Perspectives* 108(3), 463-473.

Holsapple, M.P., Morris, D.L., Wood, S.C., Snyder, N.K. (1991*).* 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced Changes in Immunocompetence: Possible Mechanisms. *Annu. Rev. Pharmacol. Toxicol* 31: 73-100.

Hoyer, A.P., Grandjean P., Jorgensen T., Brock J.W., Hartvig H.B. (1998). Organochlorine exposure and risk of breast cancer. *Lancet* 352(9143): 1816-1820.

Kakkanaiah V. N., M. Nagarkatti, and P. S. Nagarkatti. (1990). Evidence for the existence of distinct heterogeneity among the peripheral CD4-CD8- T cells from MRL-lpr/lpr mice based on the expression of the J11d marker, activation requirements, and functional properties. *Cell. Immunol.* 127(2): 442-57.

Kamath, A. B., Xu, H., Nagarkatti, P. S., and Nagarkatti, M. (1997). Evidence for the Induction of Apoptosis in Thymocytes by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin *in Vivo*. *Toxicology and Applied Pharmacology* 142: 367-377.

Kamath, A. B., Nagarkatti, P. S., and Nagarkatti, M. (1998). Characterization of Phenotypic Alterations Induced by TCDD on Thymocytes *in vivo* and its Effects on Apoptosis. *Toxicol. Appl. Pharmacol.* 150: 117-124.

Kamath, A. B., Camacho, I., Nagarkatti, P. S., and Nagarkatti, M. (1999). Role of Fas-Fas Ligand Interactions in 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Induced Immunotoxicity: Increased Resistance of Thymocytes from Fas-deficient (*lpr*) and Fas-ligand defective (*gld*) Mice to TCDD-Induced Apoptosis. *Toxicol. Appl. Pharmacol.* 160(2): 141-155.

Jensen, N. K. and G. Nordberg. (1980). Cancer patients found to have higher pesticide residues in body. *Environmental Research* 23: 257-263.

Jickells, T., A. Knap, T. Church, J. Galloway, and J. Miller. (1982). Acid rain on Bermuda. *Nature* 297: 55-57.

Johnson, P. T. J., K. B. Lunde, E. G. Ritchie, and A. E. Launer. (1999). The effect of trematode infection on amphibian limb development and survivorship. *Science* 284: 802-804.

Kaiser, J. (1998). Fungus may drive frog genocide. *Science* 281: 23.

Knap, A. H. and M-S. Kaiser (eds.) (1990). *The Long-Range Atmospheric Transport of Natural and Contaminant Substances*. Kluwer Academic Publishers. Dordrecht.

Lees, E. (1962). The incidence of helminth parasites in a particular frog population. *Parasitology* 52: 95-102.

Lin, G.L., Ellsaesser, C.F., Clem, L.W., Miller, N.W. (1992). Phorbol ester /Calcium ionophore activate fish leukocytes and induce long term cultures. *Developmental and Comparative Immunology* 16: 153-163.

Linzey, D. W. (1997). Amphibians – "Canaries in the global coal mine"? *Critter Talk*, quarterly newsletter of the Bermuda Zoological Society 20(1): 6-7.

Linzey, D. W., C. R. Bursey, and J. B. Linzey. (1998a). Seasonal occurrence of helminths of the whistling frog, *Eleutherodactylus johnstonei* (Amphibia: Leptodactylidae), in Bermuda. *Journal of the Helminthological Society of Washington* 65(2): 245-251.

Linzey, D. W., C. R. Bursey, and J. B. Linzey. (1998b). Seasonal occurrence of helminths of the giant toad, *Bufo marinus* (Amphibia: Bufonidae), in Bermuda. *Journal of the Helminthological Society of Washington* 65(2): 251-258.

Linzey, D. W., A. C. Deel, and S. J. Adams. (1998c). Spring and summer foods of *Bufo marinus* (Amphibia: Bufonidae) and *Eleutherodactylus johnstonei* (Amphibia: Leptodactylidae) in Bermuda. *The Journal of the Elisha Mitchell Scientific Society* 114(3): 125-136.

Lundberg, K. (1991). Dexamethasone and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Can Induce Thymic Atrophy by Different Mechanisms in Mice. *Biochemical and Biophysical Research Communications* 178: 16-23.

Luster, M.I., Germolec, D.R., Clark, G., Wiegand, G., Rosenthal, G.J. (1988). Selective effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and corticosteroid on in vitro lymphocyte maturation. *J. Immunol*. 140: 928-935.

Luster, M. I., Blank, J.A., Dean, J.H. (1987). Molecular and cellular basis of chemically induced immunotoxicity. *Annu. Rev. Pharmacol. Toxicol*. 27: 23-49.

Malins, D.C., Krahn M.M., Brown D.W., Rhodes L.D., Myers M.S., McCain B.B., Chan S.L. (1985). Toxic chemicals in marine sediment and biota from Mukilteo, Washington: relationships with hepatic neoplasms and other hepatic lesions in English sole (Parophrys vetulus). *J. Natl. Cancer Inst*. 74: 487.

Marco, A., C. Quilchano, and A. R. Blaustein. (1999). Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. *Environmental Toxicology and Chemistry* 18(12): 2836-2839.

McConkey, D. J., Hartzell, P., Duddy, S. K., Hakansson, H., and Orrenius, S. (1988). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Kills Immature Thymocytes by Ca^{2+}-Mediated Endonuclease Activation. *Science* 242: 256-259.

McKallip, R. J., M. Nagarkatti, P. S. Nagarkatti. (1995). Immunotoxicity of AZT: inhibitory effect on thymocyte differentiation and peripheral T cell responsiveness to gp120 of human immunodeficiency virus. *Toxicol. and Appl. Pharmacol.* 131(1): 53-62.

Nagata, S. (1997). Apoptosis by Death Factor. *Cell* 88(3): 355-365.

Nagarkatti, M., S. R. Clary, P. S. Nagarkatti. (1990). Characterization of tumor-infiltrating CD4+ T cells as Th1 cells based on lymphokine secretion and functional properties. *J. Immunol*. 144(12): 4898-905.

Nagarkatti, M. and A. M. Kaplan. (1985). The role of suppressor T cells in BCNU-mediated rejection of a syngenic tumor. *J. Immunol*. 135(2):1510-7.

Nebert, D. W. (1989). The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. *Crit. Rev. Toxicol*. 20(3): 153-74.

Pasanen, S. and P. Koskela. (1974). Seasonal changes in calcium, magnesium, copper, and zinc content in the liver of the frog, *Rana temporaria*. *Comparative Biochemistry and Physiology* 48A: 27-36.

Peterson, R.E., Madhukar B.V., Yang K.H., Matsumura F. (1979). Depression of adenosine triphosphatase activities in isolated liver surface membranes of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated rats: Correlation with effects on ouabain biliary excretion and bile flow. *J. Pharmacol. Exp. Ther.* 210(2): 275-82.

Pierce, B. A. (1985). Acid tolerance in amphibians. *BioScience* 34: 239-243.

Poland, A., and Knutson, J.C. (1982). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* 22: 517-554.

Power, T., K. L. Clark, A. Harfenist, and D. B. Peakall. (1989). A review and evaluation of the amphibian toxicological literature. *Technical Report No. 61*, Canadian Wildlife Service, Headquarters.

Powers, D. A. (1989). Fish as Model Systems. *Science* 246: 352-358.

Rhile, M. J., Nagarkatti, M., and Nagarkatti, P. S. (1996). Role of Fas apoptosis and MHC genes in 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-induced cells. *Toxicology* 110: 153-167.

Roanoke River Corridor Study Policy, Technical, and Citizens Advisory Committees. (1993). The Roanoke River Corridor Managing a Strategic Resource.

Roitt, I., Brostoff, J., Male, D. (1998). *Immunology*. Mosby, London, UK.

Russell, R. W., S. J. Hecnar, and G. D. Haffner. (1995). Organochlorine pesticide residues in southern Ontario spring peepers. *Environmental Toxicology and Chemistry* 14(5): 815-817.

Sadinski, W. J. and W. A. Dunson. (1992). A multilevel study of effects of low pH on amphibians of temporary ponds. *Journal of Herpetology* 26(4): 413-422.

Savill, J., Fadok, V., Henson, P., and Haslett, C. (1993). Phagocyte recognition of cells undergoing apoptosis. *Immunol. Today* 14, 131-136.

Shugart, L. and C. Theodorakis (1994). Environmental Genotoxicity: Probing the Underlying Mechanisms. *Environ Health Perspect.* 102(Suppl. 12): 13-17.

Silverstone, A. E., Frazier, D. E. Jr., and Gasiewicz, T. A. (1994). Alternate Immune System Targets for TCDD: Lymphocyte Stem Cells and Extrathymic T-Cell Development. *Exp. Clin. Immunogenet.* 11: 94-101.

Simmons, J. A. K. (1997). Analysis of soils for determining heavy, DCDD/F and PAHs. Unpublished report submitted to the Bermuda Ministry of the Environment.

Stancil, V. (2000). Effects of Watershed and Habitat Conditions on Stream Fishes in the Upper Roanoke River Watershed, Virginia. Master's Thesis. Virginia Polytechnic Institute and State University.

Surh, C. D., and Sprent, J. (1994). T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372**, 100-103.

Sutter, T.R., Guzman, K., Dold, K. M., and Greenlee, W. F. (1991). Targets for dioxin: Genes for plasminogen activator inhibitor-2 and interleukin-1 beta. *Science* 254: 415-418.

Thomas, P.T., and Faith, R. E. (1985). Adult and perinatal immunotoxicity induced by halogenated aromatic hydrocarbons. *Immunotoxicology and Immunopharmacology*, ed. J. H. Dean, et. al., pp.305-26. New York: Raven.

Vos, J. G., et al. (1980). Immune alterations. *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins, and Related Products*, ed. R.D. Kimbrough, pp. 241-66. Amsterdam: Elsevier.
Vos, J. G. (1977). Immune suppression as related to toxicology. *CRC Crit. Rev. Toxicol.* 5: 67-101.

Warner, S. C., J. Travis, and W. A. Dunson. (1993). Effect of pH variation on interspecific competition between two species of hylid tadpoles. *Ecology* 74(1): 183-194.

Watts, R. R. (ed.) (1980). Analysis of Pesticide Residues in Human and Environmental Samples. A Compilation of Methods Selected for Use in Pesticide Monitoring Programs. EPA-600/800-80-038. 11A:1-8.

Wester, P.W., Vethaak, A. D., and van Muiswinkel, W. B. (1994). Fish as Biomarkers in Immunotoxicology. *Toxicology* 86: 213-232.

Whitlock, J.P. (1987). The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Pharmacol. Rev*. 39(2): 147-61.

Whitlock, J.P. (1990). Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* 30: 251-77.

Curriculum Vitae
Lisa Michelle Hudson Pélanne

Education	Virginia Polytechnic Institute and State University, Blacksburg, Virginia August 1997 to April 2002 Master of Science in Immunology College of William and Mary, Williamsburg, Virginia August 1992 to May 1996 Bachelor of Science in Biology
Master's Thesis	*Use of the Immune System to Investigate the Toxicity induced by Environmental Pollutants in Fish, Amphibian, and Mammalian Species.*
Honors	Omicron Delta Kappa National Leadership Honor Society
Awards	Graduate Research and Development Program Award, May 2000
Grants Funded	Sigma Xi Grants-in-Aid-of Research, $598, March 1998 Matched by the Department of Biology, $500, March 1998
Fellowships Funded	Waste Policy Institute Graduate Fellowship, $2,500, April 1999 Matched by the Department of Biology, $500, April 1999 Waste Policy Institute Graduate Fellowship, $2,500, April 1998 Matched by the Department of Biology, $500, April 1998
Teaching Assistantships	Human Anatomy and Physiology Laboratory, 1999, 2000 Immunology Laboratory, 1999 Principles of Biology Laboratory, 1998 General Biology Laboratory, 1997 – 1998, 2000
Professional Affiliations	Virginia Academy of Sciences Society of Toxicology

Graduate Courses

Aquatic Ecotoxicology
Biochemistry for Life Sciences
Environmental Physiology of Fishes
Graduate Seminar in Microbiology/Immunology
Immunology
Introduction to Clinical Research
Molecular Biology of the Cell
Topics in Immunology
Vertebrate Microscopic Anatomy

QCA 3.77

Presentations

Hudson, L. M., M. Nagarkatti, P. S. Nagarkatti, *TCDD-Induced Apoptosis in the Thymocytes of Perinatally-Exposed Neonates*, National Meeting of the Society of Toxicology, Philadelphia, Pennsylvania, March 2000.

Sproull, M. T., A. Zeytun, M. Nagarkatti, **L. M. Hudson**, R. Duncan, P. S. Nagarkatti, *Upregulation of Fas Ligand in Various Tissues following TCDD Administration*, National Meeting of the Society of Toxicology, Philadelphia, Pennsylvania, March 2000.

Hudson, L. M., M. Nagarkatti, P. S. Nagarkatti, *TCDD-Induced Apoptosis in the Thymocytes of Perinatally-Exposed Neonates*, Graduate Research Symposium, Virginia Tech, Blacksburg, Virginia, March 2000.

Hudson, L. M., M. Nagarkatti, P. S. Nagarkatti, *Role of Apoptosis in 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced Immunotoxicity following Perinatal Exposure*, Virginia Academy of Sciences, Norfolk, Virginia, May 1999.

Hudson, L. M., M. Nagarkatti, D. Linzey, J. B. Linzey, J. Robertson, C. Brecht, J. Burroughs, J. Bacons, P. S. Nagarkatti, *Pollutant-induced Immunosuppression as a cause of Dwindling Amphibian Population*, National Meeting of the Society of Toxicology, New Orleans, LA, March 1999.

Hudson, L. M., M. Nagarkatti, D. Linzey, J. B. Linzey, J. Robertson, C. Brecht, J. Burroughs, J. Bacons, P. S. Nagarkatti, *Differences in Immune Response of Amphibians from Polluted versus Nonpolluted Environments*, Graduate Research Symposium, Virginia Tech, Blacksburg, Virginia, March 1999.

Presentations, cont.

Hudson, L. M., A. G. Heath, M. Nagarkatti, P. S. Nagarkatti, *Assessment of Proliferative and Cytolytic Activity of Lymphocytes in Fish and Their Use in Evaluation of Immunotoxicology*, Virginia Academy of Sciences, Fairfax, Virginia, May 1998.

Havill, N., **L. M. Hudson**, R. Beck**,** *Population Dynamics and Management Strategies of Beach-Nesting Birds in Virginia*, Research Symposium, College of William and Mary, Williamsburg, Virginia, 1995.

Seminars

Use of the Immune System to Investigate the Toxicity Induced by Environmental Pollutants in Fish, Amphibian, and Mammalian Species, MS Defense Seminar, Virginia Tech, April 2002.

Development of a Screening Test for the Identification of Crohn's Disease, Research and Development Seminar, TechLab, Inc., October 2001.

Adverse Effects of Environmental Pollutants on the Immune System, Microbiology and Immunology Departmental Seminar, Virginia Tech, November 1998.

Service/Leadership

Volunteer, Continuing Education Courses, Society of Toxicology, March 1999, 2000
President, Biology Graduate Student Association, 1999
President, Clayton-Grimes Biology Club, College of William and Mary, 1995-1996
Chair, Earth Day Planning Committee, College of William and Mary, 1995
Volunteer, Sierra Student Coalition, 1993-1996

- National Executive Committee Member, one of six students representing several thousand national members, 1995-1996
- Executive Chair and Chair of Conservation, William and Mary Chapter, 1993-1995
- Atlantic Coast Ecoregion Coordinator, 1994-1995

Work Experience

TechLab, Inc., Virginia Tech Corporate Research Center

- Research Scientist, August 2001-present
- Quality Control Technician, April-August 2001
- Production Laboratory Assistant, July 2000-April 2001