Effects of Selected Polycyclic Aromatic Hydrocarbons on Cell Proliferation, Protein Phosphorylation, and Intracellular Calcium Concentration in Lepomis Macrochirus.

Helen Runnels Connelly

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AROMATIC HYDROCARBONS ON
CELL PROLIFERATION, PROTEIN
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INTRACELLULAR CALCIUM
CONCENTRATION IN LEPOMIS MACROCHIRUS

A Dissertation

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in

Veterinary Medical Sciences
through the
Department of Veterinary Physiology,
Pharmacology and Toxicology

by
Helen R. Connelly
B.S., Louisiana State University, 1985
May, 1998

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Dedication

To my husband, Pete, the love of my life,

To my angels, Hannah and Cole,

And to women everywhere, who put their family first, but have a dream.
Acknowledgements

I would like to thank the people who helped with this project. These are people who have cheerfully shared their expertise, their resources, their ideas, but most importantly their time. It has been a joy to work with so many nice people.

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There is not one friend or family member that stands out in my mind as being more helpful, more supportive, or more loving than any other. I am lucky in that my friends and family are the same people. Your support, along with good luck, hard work and the grace of God, has helped me to get through this.

To my children, Hannah and Cole, who have not known me, except as a student, I love you, and I know that one day you will be so proud of your mother. You are precious angels to me, a gift from heaven. To my husband, Pete, I cannot describe what your support has meant to me. I doubt there are many women who ever experience the love I get on a daily basis, the encouragement, and the constant reminder of how they are cherished. You do this for me, and I know that it is the reason I get up every day and breathe out and in. Thank you for being the man I admire, the man who has made it possible for me to do my best, the man by my side.
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Abstract

Polycyclic aromatic hydrocarbons (PAH) have been demonstrated to affect signal transduction in higher vertebrates. In this research, the freshwater species of fish, *Lepomis macrochirus* (bluegill) was used to test the effects of a 14-day diet of PAH on lymphocyte signal transduction. PAH tested included: 2-aminoanthracene (2-AA), 2-methylnaphthalene (2-MN), 9,10-dimethylnaphthalene (9,10-DMA), and a mixture of these three compounds (MIX). Individual compounds were tested at a total PAH dose of 3.1 ± 0.01 mg over a 14 day period. The MIX diet was tested at a total PAH dose of 0.13 ± 0.01 mg 2-AA, 0.19 ± 0.01 mg 9,10-DMA, and 0.22 ± 0.02 mg 2-MN over a 14 day period. The results of these studies show that 2-AA stimulates cell proliferation. 2-MN induces some stimulatory and some inhibitory effects on cell proliferation. 9,10-DMA and MIX were demonstrated to suppress cell proliferation. Intracellular baseline calcium is reduced as a result of these PAH diets, possibly as a step prior to cell death. All PAH compounds tested are immunomodulatory to bluegill lymphocytes. The immunomodulatory effects demonstrated in this study as a result of the PAH diets are consistent with alterations to signal transduction. Specific signal transduction pathways affected by these PAH are indicated, but not specifically identified in this work.
Introduction

Fish and humans have different primary routes of exposure to environmental toxins. However, the mechanisms they use in dealing with these compounds are very similar. Fish are exposed to polycyclic aromatic hydrocarbons (PAH) primarily through the water, and humans are exposed to PAH primarily through inhalation of contaminated air and ingestion of contaminated food and water. Humans and fish, as well as rats and mice, metabolize these compounds through similar routes. All have in common the P-450 oxidation enzyme system for Phase I metabolism of compounds. Humans, rats, and fish are also capable of Phase II metabolism and excretion, or alternately, production of reactive intermediates capable of interacting with DNA and proteins. The metabolic activation of PAH to reactive intermediates, is an important step in chemical carcinogenesis, and is therefore the focus of much research. Because fish are exposed to PAH in their environment, and metabolize these compounds through routes common to humans and rats, they provide a good model for the study of chemical toxins. In this research, fish are used to study the effects of PAH on signal transduction as it relates to the immune response.

Exposure to carcinogenic PAH, such as those found environmentally in energy related waste, has been demonstrated to have immunosuppressive effects on lymphocytes (Davila et al., 1995). In fact, there is a good correlation between carcinogenicity and immunosuppression (Davila et al., 1995). Those PAH that have been demonstrated to be strong carcinogens are also likely to be strongly immunosuppressive. Three PAH were tested in this experiment for their immunomodulatory effects on lymphocytes: 2-methylnaphthalene (2-MN), 9,10-
dimethylanthracene (9,10-DMA), and 2-aminoanthracene (2-AA). Of these compounds, only 2-AA has not definitively been characterized as a carcinogen. However, all three have been characterized as mutagens.

Cellular signal transduction is a key component to activating an immune response in lymphocytes. Several biochemical pathways important to the immune response have been identified in lymphocytes. These signaling pathways are responsible for transmitting a signal from the cell surface to the nucleus, where genetic information is stored. The signal is initiated by the binding of foreign peptide antigen to the lymphocyte surface receptor. This antigen message is transmitted from the cell surface via a series or cascade of signaling molecules to the nucleus, resulting in cell activation, proliferation, and differentiation.

An appropriate cell signal is generated by the integration of several signaling pathways. At least four pathways necessary for the production of interleukin 2 (IL-2), a critical cytokine for lymphocyte proliferation, have been identified. These pathways involve the phosphorylation and activation of protein tyrosine kinases, the mobilization of intracellular calcium ([Ca^{2+}]_{i}), the activation of ras, and the activation of protein kinase C (PKC).

The phosphorylation of non-receptor protein tyrosine kinases precedes T cell activation. Within seconds of T cell receptor (TCR) binding with ligand, protein tyrosine kinases are stimulated (Samelson et al., 1986). Tyrosine phosphorylation of kinases is followed by a series of events: expression of cytokine receptors (after about 2 hours), secretion of cytokines (in about 6 hours), initiation of DNA replication (in about 24 hours), cell division (in about 48 hours), and differentiation (over a period of
days). All of these T cell responses begin with the activation of protein tyrosine kinases.

For cell activation to occur, it is necessary for several biochemical pathways to act in coordination. These pathways are complex, and remain incompletely described. The focus of this study is to increase the base of knowledge that describes the effects of PAH on these signal transduction pathways, and to describe subsequent PAH effects on immune competence. In this research, the fresh water species of fish, *Lepomis macrochirus*, is used as a model to study the effects of PAH on signal transduction and the immune response.

In order for an immune response to be amplified, subpopulations of lymphocytes undergo proliferation. Lymphocyte responses to antigens, cytokines, and antibodies can be assessed by the proliferation assay. Proliferative response of T and B cells to polyclonal mitogen stimulation is commonly used as a measure of immunologic competence. Lowered proliferative response is often a secondary effect of chronic disease.

Cell proliferation is the growth and reproduction of similar cells. It is the response to a complex set of signals initiated at the cell membrane and transduced by the coordinate action of at least four pathways: tyrosine kinase phosphorylation, PKC second messenger, Ca$^{2+}$-dependent protein activation, and ras/MAP kinase. The result of signal transduction is transcription of genes, DNA and protein synthesis, and ultimately cell division.

In this study, proliferation is used as a measure of immunocompetence. In the proliferation assay, mitogens are used to stimulate cell reproduction and lymphocyte
transformation in culture. Mitogens stimulate the cells to divide, synthesize new DNA, and take up any available nucleotides from the culture medium. Incorporated into the cell culture is a small amount of thymidine labeled with the radioactive isotope of hydrogen, tritium ($^3$H). The thymidine is incorporated only into the DNA of cells that are dividing. The incorporation of thymidine into newly synthesized DNA is representative of increases in numbers of dividing cells. The amount of radioactivity in the mitogen-treated cells is compared to the untreated cells as a reference (Tizard, 1992). The proliferation assay measures proliferation due to stimulation, which is commonly used as an indicator of immune competence.

A number of different in vitro reagents can be used to substitute for in vivo stimulation of ligand/receptor binding. Many of these stimuli polyclonally activate cells, thereby eliminating the difficulty of working with the small number of cells that are antigen specific in a larger resting population of cells. In this experiment, mitogens are used that stimulate different parts of signaling pathways, in order that affected pathways may be identified.

The mitogenic lectin from *Canavalia ensiformis*, Concanavalin A (Con A) was used in this experiment to stimulate proliferation of bluegill lymphocytes. Con A has high binding affinity for certain N-linked carbohydrates found in cell surface polypeptides, and is capable of binding many surface glycoproteins. In particular, Con A binds the three major polypeptides of the CD3 T cell surface antigen (Kanellopoulos et al., 1985). Antibodies to the CD3 T cell antigen act as polyclonal mitogens that stimulate human lymphocytes to grow and divide. T cells can be directly stimulated by interaction of lectin ligands, such as Con A, with the CD3 antigen. Because Con A
binds a part of the TCR complex (CD3) to induce cell activation, its actions mimic those of T cell with antigen, and is therefore an appropriate model for estimating immune response to antigenic challenge.

Triggering of the TCR by antigen stimulates a signaling cascade that results in cell proliferation, activation, and differentiation. This signal transduction cascade consists of tyrosine phosphorylation of proteins, activation of the calcium signal, and activation of the PKC and ras pathways. The mitogen Con A should stimulate all of these responses, because it initiates proliferation in a manner similar to TCR plus antigen. Con A binds the CD3 portion of the TCR, and is an effective mitogen for T lymphocytes. In this experiment, Con A is used as a measure of lymphocyte response to antigen.

The phorbol ester, phorbol myristate acetate (PMA), and calcium ionophore (A23187) are also tested in this experiment as stimulants to cell proliferation. Used in tandem, these pharmacologic agents stimulate T and B lymphocyte proliferation in a variety of models including murine, human, red drum, and catfish cells (Clevers et al., 1985; Burnett and Schwarz, 1994; Lin et al., 1992). PMA and calcium ionophore stimulate cell proliferation by mimicking the natural activation of PKC and/or ras. In lymphocytes, PKC binds diacylglycerol (DAG) and translocates to the plasma membrane, where it is rendered active in the presence of phosphatidylserine and Ca\(^{2+}\). As an analog of DAG, PMA is capable of binding PKC and initiating its translocation to the plasma membrane. A23187 is a calcium ionophore that increases intracellular calcium levels by mobilizing intracellular and extracellular calcium stores. Together these two agents are mitogenic for lymphocytes.
Phorbol esters activate PKC, ras, Raf-1, MEK-1, ERK-1, and ERK-2 signaling proteins (Cantrell, 1996). Because cell activation requires the coordinate action of the ras, PKC, and calcium pathways, the addition of phorbol ester alone does not serve to activate lymphocytes (Hashimoto et al., 1991). Phorbol ester plus a calcium signal or the product of activation, IL-2, is sufficient to induce cell proliferation (Hashimoto et al., 1991). The presence of monocytes (20%) in the cell culture can supply an additional signal (IL-2) which in tandem with phorbol ester can stimulate the proliferative response (Hashimoto et al., 1991).

Calcium ionophore increases intracellular calcium concentrations by mobilizing intracellular and extracellular calcium stores. This mimics the actions of inositol 1,4,5-trisphosphate (IP$_3$) in the second messenger pathway. Increased calcium in the cytosol activates calcium dependent proteins such as PKC, calmodulin and calcineurin. Calcineurin is directly responsible for activating the NFAT transcription factor, which translocates to the nucleus to bind the IL-2 gene coordinately with other transcription factors to stimulate IL-2 gene activation. Like phorbol ester, the calcium signal alone is not sufficient to stimulate cell proliferation.

In this study, the proliferation assay is used to identify steps in signal transduction that are affected by PAH. Because the different mitogens used in this experiment stimulate different parts of the signal transduction pathway, comparison of mitogen effects on PAH-treated and control cells should potentially highlight some alterations in signaling (Fig. 1). In control cells, Con A-stimulated cell proliferation is
Fig. 1 T-cell activation. Mitogens stimulate signaling proteins. Arrows indicate steps in the signaling cascade affected by the following mitogens: 1) Con A 2) calcium ionophore 3) phorbol ester.
expected to activate the cell in a similar fashion to T cell plus antigen. Therefore, all four pathways should be stimulated, resulting in proliferation. PMA-stimulated cell proliferation should activate PKC and components of the ras/MAP kinase pathway. Calcium ionophore should increase intracellular calcium and activate Ca\(^{2+}\)-dependent proteins. The combination of A23187 and PMA should be sufficient to activate cell proliferation by bypassing early protein tyrosine activation steps, mobilizing calcium, and activating PKC and ras. Therefore, each mitogen tested in this assay measures a different portion of signal transduction. Con A should stimulate all pathways and result in proliferation. A23187 should stimulate the calcium pathway. PMA should stimulate PKC and ras and their products. PMA/A23187 should activate PKC/ras and calcium, and should be sufficient to stimulate proliferation. Any alterations PAH make on these predicted responses will indicate an effect on signal transduction.

Signal transduction is dependent on intracellular calcium concentration. In response to TCR triggering, intracellular calcium is mobilized, and calcium-dependent proteins are activated. Calcium mobilization and release into the cytosol is accomplished by the binding of IP\(_3\) to an IP\(_3\) receptor on intracellular calcium stores. This generates a transient rise in cytosolic free calcium that is a signal, but is not sufficient to stimulate activation and production of IL-2. Instead, the transient rise in cytosolic calcium, or possibly the emptying of the intracellular store, triggers an influx of calcium through the plasma membrane from the extracellular milieu that is sufficient to sustain activation and production of IL-2.

The increase in cytosolic free calcium activates several calcium-dependent proteins critical to signal transduction. Some calcium-dependent proteins

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include PKC, calmodulin, and calcineurin. These proteins all play a role in cell activation. PKC regulates the NFκB transcription factor, essential to maximal activation of the IL-2 gene. Calmodulin and calcium activate calcineurin. Calcineurin is essential to the activation and nuclear translocation of the NFAT transcription factor, essential to IL-2 gene activation. In fact, the requirements for IL-2 gene activation and NFAT activation are the same, as demonstrated by the immunosuppressive agents, CsA and FK-506. CsA and FK-506, are capable of inhibiting calcineurin, which inhibits NFAT activation and translocation to the nucleus, thus inhibiting IL-2 production.

Calcium signaling, including PKC activation, calcineurin activation, and eventually IL-2 production is activated by triggering the TCR. TCR triggering initiates a cascade of events which result in the release of calcium and the initiation of calcium-dependent events. TCR stimulation initiates tyrosine phosphorylation of phospholipase Cγ1 (PLCγ1). PLCγ1 is responsible for the generation of second messengers, IP3 and DAG. IP3 is responsible for the release of Ca2+ from intracellular stores. DAG, along with Ca2+, bind and translocate PKC to the plasma membrane where it is activated. These calcium-related events are critical to the activation of the lymphocyte.

Not only is the Ca2+-signal critical to cell activation, but the concentration of intracellular free calcium in the cell has also been causally linked to the induction of apoptosis, or cell death. Prior to cell death, a Ca2+ signal may be required for the activation of an endonuclease responsible for DNA nucleosomal cleavage (Gaido and Cidlowski, 1991). Alterations in the regulation of apoptosis can
be an early event in tumorigenesis (Preston et al., 1994), however the role of Ca$^{2+}$ signaling in this process is not clear.

Alterations in intracellular calcium homeostasis have been demonstrated to play a role in PAH-induced lymphocyte immune dysfunction. Immunosuppressive PAH, such as benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA), reduce calcium mobilization and affect resting calcium levels in the cell, whereas nonimmunosuppressive PAH such as anthracene and benzo(e)pyrene (BeP) do not affect calcium levels and mobilization. In this study, the individual compounds 2-MN, 9,10-DMA, 2-AA, and a mixture of the three were tested for their effects on resting baseline calcium in bluegill lymphocytes. A change in resting or baseline intracellular Ca$^{2+}$ may be an indication of altered signal transduction, and therefore immune function in lymphocytes.

The immunomodulatory effects of a 14-day diet of PAH are measured in this study. Intracellular calcium levels and alterations in cell proliferation are used as measures of immune function and indications of signal transduction pathways affected.
Chapter 1
Literature Review

Signal transduction in lymphocytes

Signal transduction is the mechanism by which a cell translates an external stimulus to internal cellular machinery. The mechanisms involved in cell signaling are not completely understood, but several of the biochemical pathways involved have been studied. In the lymphocyte T cell model, a cell surface antigen receptor, the T cell receptor (TCR), binds the major histocompatibility complex (MHC) molecule of the antigen presenting cell. The binding of MHC complexed with peptide antigen to the TCR is typically the first step in transducing a signal. As a result of TCR engagement with antigen: a series of cellular proteins are tyrosine phosphorylated, second messengers are generated, Ca\(^{2+}\) is mobilized, protein kinase C (PKC) and ras are activated, genes are activated and transcribed, IL-2 is released, and cell proliferation is initiated. These events occurring as a result of receptor stimulation are described as signal transduction.

Structure of the T cell receptor

The cell surface antigen receptor in T cells, the TCR, is composed of multiple subunits (Fig. 2). The subunit which binds antigen complexed with MHC is made up of two chains, \(\alpha\) and \(\beta\). These peptide chains have short cytoplasmic domains of 5 amino acids. The \(\alpha\) and \(\beta\) subunits, collectively referred to as Ti, are not believed to be capable of transducing a transmembrane signal, due to their short cytoplasmic domains. The \(\alpha/\beta\) heterodimer is part of the immunoglobulin superfamily, and as

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Fig. 2. Structure of T-cell receptor and B-cell receptor.
such is able to bind a myriad of possible antigens (Dembic et al., 1986). A complex of subunits collectively referred to as CD3 is associated with the Ti antigen binding site. The CD3 is made up of four peptide chains: γ, δ, and two ε chains. These 4 chains together make up the CD3 portion of the TCR. The cytoplasmic tails of the CD3 are longer, about 40-80 amino acids (Weiss et al., 1986; Clevers et al., 1988), and are therefore candidates for participation in signal transduction. Also associated with the TCR is a dimer composed of either two ζ chains or a ζ chain in tandem with either η or the γ chain of the high affinity IgE receptor (FceRI). The ζ chain is a transmembrane protein with a large cytoplasmic domain of 113 amino acids (Weissman et al., 1988; Baniyash et al., 1989), and is believed to be a critical part of signal transduction.

**Structure of the B cell receptor**

Like the T cell, the B cell has an extracellular antigen receptor binding unit. In the B cell the antigen binding subunit is membrane immunoglobulin (mIg) (Fig. 2). The mIg is associated with a heterodimeric complex of chains comprised of Ig-α and Ig-β or Ig-γ. These chains together with the mIg are collectively referred to as the B-cell antigen receptor (BCR) (Hombach et al., 1990; Campbell et al., 1991). In humans and mice, the Ig-α and Ig-β proteins range in size from 32 to 47 kD (Baixeras et al., 1993; Cambier et al., 1993; Cambier et al., 1994). Like the CD3 and ζ subunits in the TCR, the Ig-α and Ig-β subunits in the BCR have longer cytoplasmic domains than the antigen binding mIg, which has a cytoplasmic tail of only three amino acids. The
cytoplasmic domains of Ig-α and Ig-β couple extracellular binding events to intracellular signaling machinery (Weiss and Littman, 1994).

**Protein tyrosine phosphorylation in signal transduction**

Within the cytoplasmic domains of the TCR and BCR, there exists a common sequence of peptides known as ARAM (antigen recognition activation motif) (Chan et al., 1992a; Wegener et al., 1992). The ARAM sequence consists of conserved tyrosine and leucine residues. It is this amino acid sequence that forms the critical link between receptor binding and the internal signaling cascade. The ARAM sequence by itself is sufficient to couple chimeric receptors to both early and late signaling events (Irving et al., 1993; Letourneur and Klausner, 1992; Romeo et al., 1992). It is interesting that the ARAM sequence is separate from the antigen binding molecule. That is, the Ti subunit of the TCR and the mIg of the BCR do not contain a copy of the ARAM sequence. The ARAM sequence appears in the nonantigen binding subunits of the TCR and BCR. The ARAM sequence also appears in the η subunit of the TCR and the γ subunit of the FceRI. The presence of the ARAM sequence in various signal transducing molecules and its genetic conservation underscores its importance in coupling binding events to intercellular signaling machinery.

Many cellular proteins are tyrosine phosphorylated upon antigen receptor binding in both T and B lymphocytes. The biochemical cascade of protein tyrosine kinase (PTK) phosphorylation is believed to be a critical pathway in signal transduction. There are two families of cytoplasmic protein tyrosine kinases believed to be involved in the signal transducing functions of the TCR and BCR: Src and Syk/ZAP-70. The Src family of PTK have a myristylated glycine on the N-terminal
domain that allows them to localize at the cell membrane, and a Src-homology 2 (SH2) domain that mediates recruitment of tyrosine phosphoproteins (Pawson and Gish, 1992). The Syk/ZAP-70 family of protein tyrosine kinases are not myristylated, and are therefore not likely to be localized at the plasma membrane. The Syk/ZAP-70 proteins contain two copies of the SH2 domain.

Interactions between the Src family kinases and the BCR and TCR are not well defined. In T cells there are three Src family kinases that are commonly expressed: Lck, Fyn, and Yes. The role of Lck in signal transduction of T cells is currently better defined than that of Fyn and Yes. Lck kinase activity increases after TCR stimulation (Danielian et al., 1992). However, it has been more consistently demonstrated that Lck kinase activity increases upon its association with membrane antigens, CD4 or CD8 (Veillette et al., 1991). Because CD4 and CD8 are coreceptors that bind MHC along with the TCR, it is possible that CD4 or CD8 serve to bring Lck into close proximity with the ARAM sequences in the TCR, allowing Lck to phosphorylate them. Diminished response to TCR stimulation has been demonstrated in mice deficient in Lck and T cell clones deficient in Lck (Levin et al., 1993; Molina et al., 1992; Strauss and Weiss et al., 1992). These findings indicate that the protein tyrosine kinase, Lck plays an important early role in initiating signal transduction.

In B cells, the Src-family protein tyrosine kinases that associate with the BCR include: Lyn, Blk, Fyn, and Lck (Cambier et al., 1994). As with T cells, the association seems to be between PTKs and ARAM sequences, in the cytoplasmic domain of the Ig-α and Ig-β chains. Also, analogous to T cell function, is the role that a co-receptor plays in signaling. In the B cell the co-receptor is described as
CD19/CD21 (in the T cell it is CD4 or CD8). As in the T cell, the co-receptor in the B cell may deliver a Src family PTK, Lyn, to the ARAM sequence of the Ig-α cytoplasmic portion of the BCR complex upon antigen receptor stimulation. Fyn and Lyn are both bound to the ARAM sequence in the Ig-α chain upon B-cell receptor stimulation (Okada et al., 1991). The association between PTK and ARAM may be described as either the PTK with its substrate (that is, the PTK phosphorylating tyrosines on the ARAM sequence) or the interaction between the SH2 domain of the PTK and the small amount of baseline tyrosine phosphorylated ARAMs (that is, the attraction between SH2 and tyrosine phosphorylation) (Nakayama et al., 1989; van Oers et al., 1993).

The Syk/ZAP-70 PTKs are also involved in signal transduction. Upon BCR stimulation, the Syk PTK associates with the cytoplasmic ARAM sequences of Ig-α and Ig-β (Hutchcroft et al., 1991). In T cells, antigen stimulation recruits ZAP-70 to the tyrosine phosphorylated ζ chain and CD3. A possible model for ZAP-70 involvement in TCR signal transduction is as follows: an src-family PTK, such as Lck, tyrosine phosphorylates the ARAM sequences in the ζ chain and CD3 (Chan et al., 1992b). The tyrosine phosphorylated ARAM recruits ZAP-70 via the SH2 domain in ZAP-70 (Wange et al., 1993). ZAP-70 may then be phosphorylated by a Src-family PTK (Chan et al., 1991).

CD45 is a 180-220 kd protein tyrosine phosphatase that is a necessary part of signal transduction in both T and B cells (Trowbridge, 1991). CD45 acts by dephosphorylating a negative regulatory site that exists on all Src family PTKs. By dephosphorylating the negative regulatory site, CD45 activates certain protein tyrosine
kinases such as Lck and Fyn. T cells or B cells deficient in CD45 are defective in induction of PTK catalytic function, which is an early event of TCR and BCR mediated signal transduction (Justement et al., 1991; Koretzky et al., 1990; Koretzky et al., 1991; Pingel and Thomas, 1989). The negative regulatory site on Src PTKs is initially phosphorylated by another PTK, Csk (Okada et al., 1991). Following TCR-stimulated tyrosine phosphorylation of proteins, are several integrated signal transducing biochemical pathways.

Comparison of T and B signaling pathways

T and B cells have different effector functions and recognize different antigens. However, their signal transduction components are remarkably similar. They both have oligomeric receptors containing separate antigen binding and signal transducing molecules. Both B and T cells use coreceptors to stabilize cell/antigen interactions. The coreceptors may also function to bring Src family proteins into proximity of the TCR or BCR to phosphorylate tyrosine substrates on ARAM sequences. Both B and T cells have ARAM sequences in the cytoplasmic tails of their signal transducing molecules. The ARAM sequences are phosphorylated by Src-family PTKs, creating high affinity binding sites for Syk in B cells and ZAP-70 in T cells. Also, Syk/ZAP-70 PTKs associate with phosphorylated ARAM sequences in both cells types. Syk and ZAP-70 are so similar that in B cells, Syk function can be reconstituted by ZAP-70 (Kong et al., 1995). Upon binding the ARAM sequence, Syk and ZAP-70 kinase function is increased. In both B and T cells, CD45 functions as a phosphatase inhibitor. The presence of this protein is essential in T and B lymphocytes for receptor-mediated events to occur. Phospholipase C (PLC) is
activated by tyrosine kinase activity in both T and B cells. PLC activation in T and B lymphocytes results in the generation of second messengers, and the subsequent rise of intracellular calcium. Because of the similarities in the signaling pathways in B and T lymphocytes, conclusions can be drawn concerning both populations of cells. In the present research, T and B lymphocytes are not separated, and only conclusions appropriate for both populations are drawn.

The events following protein tyrosine phosphorylation, linking TCR stimulation to IL-2 gene transcription are still under investigation. Downstream of protein tyrosine phosphorylation are three fairly well described signaling pathways that synergize to couple the TCR to gene transcription in the nucleus. These pathways involve the activation of 1) protein kinase C, 2) calcium/calcineurin, and 3) ras. The final target of each pathway is nuclear transcription factors. The coordinate action of multiple transcription factors is necessary for the regulation of IL-2.

**Phosphatidylinositol (PI) second messenger pathway: activation of PKC and release of intracellular calcium**

The activation of the PI second messenger pathway follows PTK phosphorylation and stimulates the release of intracellular calcium and the activation of PKC (Fig. 3). The PI second messenger pathway is initiated by TCR-induced PLCγ1 activation (Mustelin et al., 1990). Tyrosine phosphorylation and activation of PLCγ1 results in hydrolysis of PI 4,5-bisphosphate, which yields second messengers: inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Second messenger, IP₃ is responsible for the TCR-induced rapid increase in [Ca²⁺]. IP₃ binds a specific receptor (IP₃R) on the endoplasmic reticulum, and through a conformational
Fig. 3 Phosphatidylinositol second messenger pathway (PKC).
change, \( \text{Ca}^{2+} \) is released from intracellular stores into the cytoplasm (Crabtree, 1994). Second messenger, DAG and \( \text{Ca}^{2+} \) are responsible for activation of PKC, a serine/threonine kinase. Increased \([\text{Ca}^{2+}]_i\) and activation of PKC have been linked to cellular responses in T and B cells, the best characterized of which is the transcriptional activation of the IL-2 gene. PKC may also phosphorylate a variety of proteins on serine and threonine. The mechanism for tyrosine phosphorylation of PLC\(\gamma 1\) by the TCR is not clear, however Lck may directly or indirectly be responsible for phosphorylating PLC\(\gamma 1\) (Strauss et al., 1992).

**The calcium pathway**

The increase in \([\text{Ca}^{2+}]_i\), resulting from PLC activation, is important in calcium/calmodulin-dependent events occurring downstream of the \([\text{Ca}^{2+}]_i\) increase (Fig. 4). The calcium signal is necessary for production of IL-2. A 1-2 hour elevated \([\text{Ca}^{2+}]_i\) concentration is required for the production of IL-2. Although transcription of the IL-2 gene is detected within 40 minutes of contact with antigen, a sustained elevated calcium level is necessary for the maintenance of transcription.

Calcineurin, a calcium/calmodulin-dependent serine phosphatase, is activated downstream of the \([\text{Ca}^{2+}]_i\) increase. The increase in calcium leads to the activation of calmodulin, which activates calcineurin by exposing its active site. Calcineurin is critical in activating NFAT (nuclear factor of activated T cells), an IL-2 gene transcription factor. Activated calcineurin activates cytosolic NFAT transcription factor by dephosphorylating it on multiple serine residues. Dephosphorylation of NFAT increases its binding affinity for DNA and causes it to relocate to the nucleus.
Fig. 4 Calcium pathway.
The transient (approximately one minute) increase in cytosolic calcium generated by
the binding of IP₃ to intracellular stores (Crabtree and Clipstone, 1994) is not sufficient
to keep NFAT activated and localized in the nucleus. Rather, the initial increase in
intracellular calcium may serve as a signal for the influx of capacitive calcium across
the plasma membrane. In resting cells, the cytosolic [Ca²⁺] concentration is
approximately 100nM, whereas the concentration in the endoplasmic reticulum and
the extracellular millieu is in the millimolar range. These calcium sources supply the
sustained calcium level necessary for the NFAT transcription factor to remain
activated and compartmentalized in the nucleus. Once in the nucleus, NFAT binds the
IL-2 gene cooperatively with another transcription factor, AP-1.

The activated ras pathway

The AP-1 transcription factor, which cooperatively binds the IL-2 gene with
NFAT, is the target of another signaling pathway activated upon TCR stimulation, the
activated ras pathway (Fig. 5). The quantity of GTP-bound ras (activated ras)
increases immediately upon TCR stimulation. The ras genes constitute a family of
genes that code for 21-kDa proteins localized at the inner side of the plasma
membrane. The increase in activated ras may occur through TCR inhibition of GAP
(guanine nucleotide exchange protein) or TCR activation of another GTP exchange
protein, Sos. Sos may couple the activated TCR to ras via a complex of proteins:
p36/Grb2/Sos. It is possible that activation of ras is accomplished through both
mechanisms: inhibition of GAP and the coupling of ras to the TCR via p36/Grb2/Sos.
Activated ras directly binds Raf-1. Once bound, Raf-1 translocates to the plasma
Fig. 5 Ras/MAP kinase cascade.
membrane where it is phosphorylated and activated. The phosphorylation of Raf-1 is accomplished via PKC, lck, or possibly another kinase.

The pathway of sequentially phosphorylated proteins that follows the activation of Raf-1 is known as the mitogen activated protein (MAP) kinase pathway due to the large number of mitogens that can stimulate the pathway. It is also known as the extracellular regulated kinase (ERK) pathway for the same reason. The first step in the pathway involves Raf-1 activation of MEK, a dual specificity kinase. Raf-1 phosphorylates MEK on serine, thus activating it (Seger and Krebs, 1995). MEK, a dual specificity kinase, phosphorylates MAP kinases ERK-1 and -2 first on tyrosine and then on threonine (Seger and Krebs, 1995). ERK-1 and -2 are so similar that they are considered to be functionally redundant. ERK kinases have numerous cytosolic and nuclear substrates, and are able to translocate to the nucleus to directly phosphorylate transcription factors. Substrates for ERKs are serine or threonine residues neighboring proline. In the nucleus ERK phosphorylates a transcription factor complex, SRF/Elk-1 (serum response factor/Elk-1). This complex binds the c-fos SRE (serum response element) regulatory sequence to stimulate transcription of c-fos (Hunter and Karin, 1992; Angel and Karin, 1991; Marais et al., 1993). Similarly, ERK or JNK serine phosphorylate and augment the transcriptional activity of c-jun to induce c-jun (Angel and Karin, 1991). Proteins from the fos and jun family form dimers which are known as AP-1 (activator protein) (Angel and Karin, 1991). AP-1 proteins participate in the formation of NFAT and NF-IL-2 transcription factors (Angel and Karin, 1991). AP-1 and NFAT bind adjacent sites on the IL-2 promoter.
and enhancer. IL-2 promoter activation requires cooperative interaction of several transcription factors including AP-1, NFAT, NFκB and NF-IL-2 (Angel, 1991).

The ras pathway is essential but not sufficient to stimulate the transcription of IL-2. The ras/MAP kinase pathway (ras, Raf-1, MEK, ERK, Elk-1/SRF, c-fos and c-jun) results in the formation of AP-1 through the dimerization of fos and jun. AP-1 is essential to IL-2 induction. Although the MAP kinase pathway/AP-1 formation is independent of and not stimulated by calcium, the synergistic action of calcium signaling is required for IL-2 transcription. The ras pathway synergizes with signals from PKC and calcium to activate the IL-2 gene. Calcium ionophore and PMA can stimulate IL-2 transcription by activating PKC, ras, and calcium release. However, the production of IL-2 requires the action of all of these pathways. The activation of lymphocytes requires the coordinate action of several biochemical pathways, beginning with TCR stimulation and PTK activation and ending with several signals combining to induce IL-2, which is encoded by a single gene.

**PAH as immunotoxins**

Polycyclic aromatic hydrocarbons (PAH) are widespread environmental pollutants. The primary source of PAH pollution in our environment is the incomplete combustion of fossil fuel for power generation, heat, and transportation. The major source of atmospheric PAH in the United States, at least 36%, is thought to be from motor vehicles (Lee et al., 1995). Other sources of PAH pollution include cigarette smoke and industrial processes. Humans and animals are exposed to PAH primarily
through inhalation of polluted air and ingestion of contaminated food and water (Zedeck, 1980).

Many PAH are carcinogenic (Dipple et al., 1976; Zedeck, 1980). Benzo(a)pyrene (BaP), one of the best studied carcinogenic PAH, exerts its carcinogenic effects by metabolism to reactive diol epoxide intermediates, which subsequently bind DNA or cellular proteins to form adducts (Nebert, 1987). This process is initiated when a PAH binds the cytosolic aromatic hydrocarbon receptor (AhR) (Bradfield et al., 1990), which triggers the activation of Phase I metabolic enzymes. Cytochromes P-4501A1 and P-4501A2 are primarily responsible for bioactivating PAH into ultimate carcinogens (Nebert, 1987).

Many carcinogenic PAH are immunosuppressive, while their noncarcinogenic analogs are not immunosuppressive (Dean et al., 1983; Malmgren et al., 1952). For example, BaP and 7,12-dimethylbenz(a)anthracene (DMBA) are both carcinogenic and immunosuppressive. Whereas, benzo(e)pyrene (BeP) and anthracene are relatively noncarcinogenic and nonimmunosuppressive. Because PAH carcinogenicity is mediated by P-450 biotransformation, it was originally suggested that PAH immunomodulation might also be mediated by P-450 metabolic activation. There is now evidence that suggests that although enzyme mediated metabolism may be a part of PAH-induced immunosuppression, it is not the only mechanism involved (White et al., 1985; Thurmond et al., 1987; Thurmond et al., 1988).

There is a growing body of evidence that suggests that PAH-mediated immunosuppression may be due to alteration of cellular signal transduction pathways. Specifically, PAH may interfere with critical parts of signaling pathways including
alteration of intracellular \([\text{Ca}^{2+}]\) homeostasis and/or cellular protein tyrosine phosphorylation and activation (Davila et al., 1985). In this model of immunosuppression, PAH alter immune function by altering signal transduction that follows cell stimulation. In this way, PAH would affect the results of signal transduction: transcription of genes, production of proteins, and ultimately cell division.

PAH induce immunotoxic responses in lymphoid cells and organs. Doses of PAH higher than needed to induce immunotoxicity are often cytotoxic to lymphoid cells. Lower doses of PAH may induce immunotoxicity without overt cytotoxicity (Ward et al., 1984; Burchiel et al., 1990). In general, immunotoxic PAH may induce a variety of responses including lymphoid organ atrophy, lymphoid cell death, apoptosis (Burchiel et al., 1992; Burchiel et al., 1993), decreased T and B cell response to mitogens, and decreased cell-mediated response (Ward et al., 1985; Dean and Adams, 1985; White, 1986; Luster and Blank, 1987). In the present research, we examine the effects of immunotoxic PAH at doses lower than necessary to induce cell death, but in a range high enough to induce immunotoxicity.

**The role of P-450 metabolic activation in PAH-induced immune suppression**

The best characterized immunotoxic compounds are BaP and DMBA. These two carcinogenic PAH are both immunosuppressive, but seem to have slightly different mechanisms of action (Bigelow and Nebert, 1982; Wodjani and Alfred, 1984a; Thurmond et al., 1988). In order to determine if the immunosuppressive effects of these two compounds are mediated by P-450 metabolism, experiments were
done using Ah-responsive and Ah-nonresponsive mice. DMBA was found to suppress both humoral and cellular immune function in Ah-responsive and Ah-nonresponsive mice, *in vivo* and *in vitro* (White *et al.*, 1985; Thurmond *et al.*, 1987; Thurmond *et al.*, 1988). This indicates that DMBA either bypasses the P-450 activation pathway, uses another metabolic activating pathway, or has a direct effect on immune cells. BaP was found to be more immunosuppressive in Ah-responsive mice than in Ah-nonresponsive mice (Wojdani *et al.*, 1984b; Thurmond *et al.*, 1988), which indicates that metabolism may play a role in BaP-induced immunosuppression. However, BaP does cause a measurable amount of immunosuppression in Ah-nonresponsive mice (Wojdani *et al.*, 1984b; White *et al.*, 1985), which suggests that BaP-induced immunosuppression may be caused by mechanisms in addition to P-450 activation metabolism. It has recently been shown that the Ah receptor (AhR) does not play a role in all forms of immunotoxicity. Fluoranthene can induce apoptosis in a clearly AhR-deficient T cell hybridoma cells (Yamaguchi *et al.*, 1996). It has also recently been demonstrated that the affinity of a compound for the Ah receptor is not directly related to PAH immunotoxicity. For example, the highly immunotoxic PAH, BaP and DMBA have lower binding affinities for the AhR than benz(a)anthracene, which is only mildly immunotoxic (Davila *et al.*, 1996). These facts together demonstrate that while binding the AhR and P-450 metabolism may be involved in PAH-mediated immunotoxicity, AhR binding does not play an obligatory role in all forms of immunotoxicity.

In experiments using α-naphthoflavone, a P-450 inhibitor, suppressive effects of DMBA on *in vitro* humoral immunity can be partially reversed. However, α-
naphthoflavone does not inhibit DMBA-induced cell-mediated immune response (measured as T-cell proliferation) (Thurmond et al., 1989). These results indicate that P-450 may play a role in DMBA-induced humoral immune suppression, but only a minor role in cell-mediated immune function. BaP-mediated humoral and cellular immune suppression is partially inhibited by α-naphthoflavone (Kawabata, 1987; Mudzinski, 1993). These results suggest that P-450 metabolic activation may have more of a role in BaP-induced immunosuppression than in DMBA-induced suppression.

DMBA and BaP appear to have slightly different mechanisms of action in mediating immune suppression (Bigelow and Nebert, 1982; Wodjani and Alfred, 1984a; Thurmond et al., 1988). To summarize: DMBA can induce immune suppression in Ah-responsive and Ah-nonresponsive mice, and DMBA-induced humoral but not cell-mediated immune suppression can be partially reversed by using the P-450 inhibitor, α-naphthoflavone. These facts together suggest that P-450 metabolic activation may play a role in DMBA-induced humoral, but not cellular immune response. Possible explanations for these findings include the following: murine splenocytes do not metabolize DMBA to an appreciable extent (Thurmond et al., 1989), and DMBA metabolites are not much more immunosuppressive in vitro than the parent compound (Thurmond et al., 1989). In the case of BaP: BaP is more immunosuppressive in Ah-responsive than Ah-nonresponsive mice, although BaP does induce a measureable amount of immune suppression in Ah-nonresponsive mice. α-Naphthoflavone can partially inhibit both humoral and cell-mediated in vitro immune suppression induced by BaP. These observations suggest that BaP-induced
immune suppression is more dependent on P-450 metabolic activation than is DMBA-induced suppression, however, mechanisms other than P-450 activation are likely to be involved in BaP-induced immune suppression. It is likely that the immunomodulatory effects of BaP, DMBA, and other PAH are mediated by a combination of direct and P-450-dependent mechanisms.

**PAH effects on humoral immunity**

Humoral immune responses appear to be more sensitive to PAH modulation than do cellular responses (Luster, 1992). The primary function of the humoral branch of the immune system is to rid the body of extracellular pathogens, by way of B cells producing specific antibodies to antigen. This response is dependent on help from T lymphocytes, and is therefore considered T-dependent. There are also humoral T-independent responses which are assessed by B-cell specific mitogens.

DMBA and BaP may suppress humoral immunity using slightly different mechanisms of action. DMBA suppresses T-independent and T-dependent B-cell response in vivo and in vitro (Stjernswärd, 1966; Baroni et al., 1975; Yamashita et al., 1982a; Ward et al., 1984; Ward et al., 1986; Dean and Adams, 1985; White et al., 1985; Thurmond et al., 1987; Thurmond et al., 1988; Burchiel et al., 1988; Ladies et al., 1991). BaP suppresses T-dependent response after in vitro or in vivo exposure (Stjernswärd, 1966; Dean et al., 1983; White and Holsapple, 1984; Lyte and Bick, 1985; White et al., 1985; Blanton et al., 1986; Kawabata and White, 1987; Ladies et al., 1991; Ladies et al., 1992; Tomar et al., 1991). BaP suppresses B cell response to certain T-dependent antigens after in vitro exposure (Dean et al., 1983; Ladies et al., 1992), but BaP exposure in vivo or in vitro has no effect on B-cell response to the

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polyclonal T-independent mitogen, LPS (Ladies et al., 1992; Tomar et al., 1991). Because BaP suppresses T-dependent responses, but not T-independent response to the polyclonal mitogen LPS, it is possible that the target of BaP-induced humoral suppression is not the B cell itself, but other cells such as the antigen-presenting macrophage (Ladies et al., 1992). Another possibility is that PAH do not exert effects on the LPS signaling pathway, a calcium-independent response. However, other experiments suggest that B-cells, not T-helpers or macrophages are the direct target of BaP-induced suppression (Dean et al., 1983). The same disparity exists in results from DMBA experiments, some suggest that antigen presenting macrophages are the target (Yamashita and Hamaoka, 1982a), others suggest that the B-cell itself is the direct target (Ward et al., 1984; Davis and Burchiel, 1992).

In vivo exposure to other carcinogenic compounds such as benz[a]anthracene, dibenz[a,c]anthracene, dibenz[a,h]anthracene, and 3-methylcholanthrene cause decreased response to T-dependent antigens (Malmgren et al., 1952; Stjernswärd, 1966; Lubet et al., 1984; White et al., 1985). Less carcinogenic or noncarcinogenic PAH such as anthracene, chrysene, BeP, phenanthrene, and perylene do not suppress antibody response in this system.

The target of PAH-induced humoral suppression is not clear. The target may be the T-helper lymphocyte, the antigen-presenting macrophage, or the B cell itself. It is likely that the immunomodulatory effects of BaP, DMBA, and other PAH on humoral immune response are due to both direct and indirect mechanisms.
PAH effects on cell mediated immunity

The cell-mediated branch of the immune system is responsible for ridding the body of intracellular pathogens, virus-infected cells, tumor cells, and foreign grafts. T cells, macrophages, and natural killer cells all participate in cell-mediated responses. DMBA suppresses all types of cell-mediated immunity: T cell proliferative response to Concanavalin A (Con A) and phytohaemagglutinin (PHA), cytotoxic T lymphocyte (CTL) activity, natural killer cell activity, and mixed lymphocyte response (MLR) in vitro (Yamashita et al., 1982b; Ehrlich et al., 1983; Dean and Adams, 1985; Dean et al., 1986; Ward et al., 1986; House et al., 1987b; Cornacoff et al., 1988; Thurmond et al., 1989; Wood and Holsapple, 1993). DMBA decreases delayed-type hypersensitivity response in vivo (Yamashita et al., 1982), host resistance to tumor challenges in vivo (Ward et al., 1984; Dean and Adams, 1985), and graft versus host and graft rejection (DiMarco et al., 1971).

The effects of BaP on cell-mediated immunity are not as uniform as are the effects of DMBA. Both suppression and an increase in proliferation have been reported with in vitro BaP exposures to murine cells stimulated with the T-cell mitogen, Con A (Tomar et al., 1991; Thurmond et al., 1988). Both suppression and no effect have been reported with murine cells exposed in vivo and in vitro to BaP in response to the T-cell mitogen, PHA (Dean et al., 1983; Wojdani and Alfred, 1984; Tomar et al., 1991). BaP suppresses the MLR in murine spleen cells when incubated in vitro (Thurmond et al., 1988), while in vivo BaP exposure causes no change in the MLR (Dean et al., 1983). Limited work in human lymphocytes shows suppression in Con A and PHA stimulated lymphocytes (Mudzinski, 1993; Pallardy et al., 1992;
Krieger et al., 1995; Davila et al., 1995) when incubated in vitro with BaP. The meaning of these results is not clear. BaP appears to have an inconsistent effect on cell-mediated immunity as compared to DMBA, which uniformly causes immunosuppression. BaP appears to have a greater effect on humoral immunity, but also affects certain aspects of cell-mediated immunity. The apparent differences in the effects of DMBA and BaP on humoral and cellular immunity make further investigation into the mechanism of immunosuppression an active ongoing area of research.

**Effects of PAH on cytokine production**

One of the mechanisms by which PAH may exert effects on cell-mediated immunity is inhibition of cytokine secretion or cytokine receptor expression. Both BaP and DMBA have been demonstrated to have similar effects on IL-2 regulation. Both BaP and DMBA suppress the secretion of IL-2 and expression of the IL-2 receptor in murine lymphocytes after in vitro incubation (House and Dean, 1987a; Pallardy et al., 1988; Thurmond et al., 1988; Lyte and Bick, 1987; Myers et al., 1988). Addition of exogenous IL-2 can reconstitute DMBA-impaired cytotoxic T lymphocyte activity in vitro (Dean and Adams, 1985; House et al., 1987b), and can reconstitute BaP-impaired T-dependent antibody response (Lyte et al., 1987). Reconstitution of function after addition of exogenous IL-2 suggests that PAH exert their suppressive effects on T helper cells which normally secrete IL-2. This may be a direct or indirect effect on T cells. IL-1 secretion by macrophages may also be a target of BaP. Addition of IL-1 reconstitutes BaP-induced suppression of antibody production (Lyte and Bick, 1986). These studies suggest that alteration of cytokine secretion and receptor expression may be a primary mechanism of PAH immunomodulation.
Effects of PAH on lymphocyte signaling: mechanisms of immunotoxicity

The effects of PAH on immune function have been well described, but the mechanisms by which they exert these effects are less well understood. It is possible that PAH exert immunomodulatory effects by disrupting cellular activation pathways. Normally, cells respond to receptor stimulation with a complex set of signals, the result of which is cell activation and proliferation. It is possible that PAH interfere with this signaling pathway to create immune dysfunction.

One mechanism by which PAH may disrupt lymphocyte immune function is by altering $[\text{Ca}^{2+}]_i$, homeostasis in the cell. Exposure of T and B lymphocytes to DMBA, in vivo or in vitro, decreases $[\text{Ca}^{2+}]_i$ mobilization after mitogen stimulation and increases resting or baseline $[\text{Ca}^{2+}]_i$ levels in cells (Burchiel et al., 1990; Burchiel et al., 1991; Burchiel et al., 1992; Davis and Burchiel, 1992). These effects are seen in $\text{Ca}^{2+}$-dependent pathways of lymphocyte activation. $\text{Ca}^{2+}$-independent pathways of activation do not appear to be affected by exposure to DMBA. For example, IL-4 induced Class II major histocompatibility complex (MHC) antigen expression in B cells, a $\text{Ca}^{2+}$-independent event, is unaffected by DMBA exposure (Davis and Burchiel, 1992; Burchiel et al., 1992). Nonimmunosuppressive PAH such as BeP and anthracene do not alter $[\text{Ca}^{2+}]_i$ homeostasis in this model (Burchiel et al., 1991).

PAH effects on T lymphocyte signal transduction

As described previously, TCR-mediated activation of the T cell results in rapid tyrosine phosphorylation of cellular proteins, a rapid increase of intracellular $[\text{Ca}^{2+}]_i$, and phosphorylation and activation of the ras/MAP kinase pathway. PAH may
interfere with protein phosphorylation events and $[\text{Ca}^{2+}]_i$ homeostasis in the T cell, and thereby interfere with normal signal transduction. PAH have been shown to cause an upset in cellular calcium homeostasis in a structure-related fashion. The more carcinogenic and immunosuppressive PAH have been shown to stimulate a sustained increase in intracellular $\text{Ca}^{2+}$ levels, possibly by inhibiting the reuptake of $\text{Ca}^{2+}$ by smooth endoplasmic reticulum ATP-ases (SERCA). PAH that are neither carcinogenic or immunosuppressive cause only a transient rise in intracellular $\text{Ca}^{2+}$ (Krieger et al., 1995). For example, in the HPB-ALL human T cell line, the highly immunosuppressive PAH, BaP and DMBA, and the moderately immunosuppressive PAH dibenzo[\text{a, h}]-anthracene and 9,10-dimethylanthracene produce a sustained $[\text{Ca}^{2+}]_i$ elevation for at least 4 hours. Minimally immunosuppressive PAH such as BeP and anthracene cause only a transient (3 minute) rise in intracellular calcium (Mounho et al., 1997). In this same model, the metabolites of BaP, 7,8-dihydrodiol and 7,8-diol-9,10-epoxide were more effective than parent BaP in increasing intracellular $\text{Ca}^{2+}$, suggesting a role for P-450 metabolism in BaP-induced immunotoxicity. A prolonged increase in $[\text{Ca}^{2+}]_i$ caused by metabolites of BaP, but not BaP itself, has been shown to cause a depletion of intracellular glutathione (Romero et al., 1997). While glucuronidation pathways are the primary Phase II pathways for detoxification and inactivation of BaP metabolites, glutathione may also be consumed in this process. The fact that the metabolites of BaP, but not BaP itself cause a reduction in cellular glutathione is evidence that P-450 metabolism may be involved in PAH-induced immunotoxicity. One of the roles of glutathione in the cell is to buffer the effects of reactive oxidants, such as epoxides, on sulfhydryl oxidation.
The decrease in glutathione levels associated with prolonged \([\text{Ca}^{2+}]_{i}\) increase, may be the result of oxidant species attack on sulfhydryl-sensitive proteins (SERCA, PKC, and protein tyrosine phosphatases) involved in \(\text{Ca}^{2+}\) signaling. Altered calcium concentrations within the cell, especially at elevated levels, may be a key component in PAH-induced immune suppression.

In a human T cell line, DMBA has been shown to activate the protein tyrosine kinases Fyn and Lck, and to increase tyrosine phosphorylation of PLCγ1 (Archuleta et al., 1993). DMBA-induced protein tyrosine kinase activation may also be responsible for producing an early and sustained rise in intracellular calcium in a human T cell line (Krieger et al., 1994). The effects of PAH on other signaling proteins such as MAP kinase, and PKC have not been studied. These investigations of how PAH affect the activation of these proteins is an important area of new research.

**PAH effects on B lymphocyte signal transduction**

DMBA produces a sustained rise in intracellular calcium in both a murine B-cell lymphoma A20.1 cell line and murine splenic B cells (Davis and Burchiel, 1992; Burchiel et al., 1993). This is comparable to the effects of DMBA on human T cells. DMBA also increases tyrosine phosphorylation in B cells. Incubating DMBA with A20.1 murine B cells results in increased tyrosine phosphorylation of proteins (Davila et al., 1995). However, carcinogenic and noncarcinogenic PAH were both able to stimulate increased protein phosphorylation in this model. The proteins phosphorylated upon incubation with DMBA were associated with the mIg complex of the B cell. This suggests that DMBA may alter signal transduction at the level of the BCR. Although signaling pathways in the B cell have not been as extensively

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studied as in the T cell, it is possible that PAH act at analogous sites in T and B cells. Further investigation into the effects of PAH on signaling protein activation is necessary to determine where in the signaling pathway immunomodulation occurs. This is interesting not only as it concerns PAH-induced immune dysfunction, but as a step towards understanding signal transduction.

**Teleost immune system**

In this study, fish are used to investigate the effects of PAH on immune function, and in particular, the effects of PAH on signal transduction. Fish are a desirable model for the study of effects of PAH on immune response because the immune system of teleosts is functionally similar to that of higher vertebrates. They exhibit the overall characteristics of both cellular and humoral immunity: allograft rejection (Hildeman, 1958; Miller *et al.*, 1986; Caspi and Avtalion, 1984), processing and presentation of antigen (Vallejo, 1991), vigorous mixed lymphocyte reaction (MLR) (Miller *et al.*, 1986), generation of antibodies to specific antigen, and a heterogenous lymphocyte population. At the molecular level, there is also correlation between mammalian and teleost immune function. The humoral immunoglobulin of teleosts is a tetrameric molecule (van Ginkel *et al.*, 1991) homologous to human IgM (Wilson and Warr, 1992). Recently cloned teleost genes include: TCR-β in rainbow trout (Partula *et al.*, 1995) and MHC Class I and II in carp, Atlantic salmon, rainbow trout, and zebrafish (Hashimoto *et al.*, 1990; Grimholt *et al.*, 1993; Glamann, 1995; Sultmann *et al.*, 1994; Takeuchi *et al.*, 1995), respectively. The most notable difference between the teleost and mammalian immune system is the absence in fish
of lymph nodes and the presence of an anterior or head kidney, an analog to bone marrow in mammals.

In mammals, lymphocytes fall into two categories: T cells, which are involved in cell-mediated immunity and B cells, which produce antibody. T cells generally protect the host from intracellular pathogens and B cells protect the host from extracellular pathogens. These two cell populations are identifiable by their cell surface markers and by certain functional differences. B and T cells can be characterized by their response to certain mitogens. For example, T cells proliferate in response to the mitogen Con A and B cells respond to LPS. T cells can also be identified by their proliferative response to the MLR assay. Within each category of lymphocytes there are subdivisions. T cells are divided into categories identified by surface antigens CD4 and CD8. B cells are divided by immunoglobulin class.

Fish lymphocytes demonstrate heterogeneity resembling that of mammals. In the trout, thymus cells respond exclusively to T mitogens (Con A), lymphocytes in the head kidney respond exclusively to B mitogens (LPS) and lymphocytes in the spleen respond to both (Tomonaga et al., 1973). Lymphocyte activation in peripheral blood has been demonstrated by the MLR in catfish (Miller et al., 1986), trout (Kaastrup et al., 1988), and carp (Caspi and Avtalion, 1984).

Catfish peripheral blood lymphocytes can be separated into functionally distinct subpopulations of lymphocytes using monoclonal antibodies (mAb) to serum immunoglobulin. This effectively identifies the Ig^+, or B cells (Secombes, 1994). T cell (Ig^+) surface markers reactive with mAb have been identified in channel catfish (Miller et al., 1987; Passer et al., 1996).
Peripheral blood lymphocytes from the freshwater species of fish, *Lepomis macrochirus* (bluegill), are used in this research. Bluegill were chosen for this study due to their abundance in environments affected by PAH (Carlander, 1977), and for their adaptability to the lab environment. It has previously been established that bluegill lymphocytes from peripheral blood and the anterior kidney exhibit both T and B cell characteristics (Cuchens and Clem, 1977; Smith *et al.*, 1967; Kusher and Crim, 1991). Bluegill have at least two populations of lymphocytes: one that responds to PHA (T-like) and one that responds to LPS (B-like). Because there are not currently monoclonal antibodies to surface markers on T and B bluegill lymphocytes, the lymphocyte populations in this research are not separated. The conclusions drawn thus apply to both T and B populations of lymphocytes, and contribute to the general understanding of signal transduction. In many instances in this study, explanations of signal transduction are described in terms of T cell signaling, because those pathways have been better characterized. Additionally, the T cell mitogen, Con A, is used for lymphocyte stimulation in this study. However, conclusions are only drawn that apply to both T and B cell signal transduction. Signal transduction in T and B lymphocytes differs at the protein subunit level, but the biochemical pathways involved in transducing the signal are remarkably similar. As previously described, signal transduction in both T and B lymphocytes involves phosphorylation of proteins, second messenger generation, and Ca$$^{2+}$$-dependent mechanisms.

**Effects of PAH on fish immunity**

Fish provide a good model for studying immunotoxic effects of PAH in the environment, due to similarities between fish and mammalian response to PAH.
Because they are exposed via the aquatic environment to PAH pollution, there have been several investigations into PAH-induced effects on fish. Fish living in aquatic environments with high concentrations of PAH suffer from numerous pathological conditions including eye lens cataracts (Hargis and Zwerner, 1988a), gill necrosis (Hargis and Zwerner, 1988b), necrosis of the renal epithelium (Thiyagarajah et al., 1989), and neoplasms (Hargis et al., 1989). Fish from PAH-contaminated waters and PAH exposures in the lab, demonstrate DNA adduct formation (Stegeman and Kloeper-Sams, 1987). Fish, like higher vertebrates, metabolize PAH such as BaP to reactive intermediate species (Steward et al., 1989), using the P-450 biotransformation pathway (Stegeman and Kloeper-Sams, 1987). The primary route of excretion of PAH and metabolites in fish is from the liver to the gastrointestinal tract via the bile, from the kidney into the urine, and through the skin where the compounds are bound to mucus (Varanasi et al., 1978). Fish respond to PAH in much the same way as mammals. They either biotransform PAH to excretable metabolites or they form reactive intermediates leading to DNA adduct formation, tumor formation, or possibly comprised immunocompetence.

PAH-mediated immune dysfunction like that found in mammals is also documented in fish. PAH effects on mammalian lymphocytes do not seem to be consistent across labs, lymphocyte populations, or compounds used. The same is true in fish studies. Both suppression and proliferation of both T and B lymphocyte response is reported.

In teleost T lymphocyte assays both enhanced and suppressed cell proliferation have been reported. Suppression of Con A and PHA-stimulated lymphoproliferation
due to PAH exposure in contaminated waterways and in vitro lab exposures has been reported in spot fish (Leiostomas sp.) (Faisal and Huggett, 1993; Faisal et al., 1991a). Other reports demonstrate enhanced T cell proliferation as a result of exposure to a mixture of PAH via injection or through aquarium exposure (Tahir et al., 1995; Arkoosh et al., 1996).

In B cells there are also reports of both suppression and proliferation. Dab exposed to increasing amounts of diesel based drilling mud in their aquaria and spot collected from increasingly more PAH-contaminated environments both demonstrate increasing B cell responses as the sediment PAH load increases. The responses measured were number of antibody secreting cells and response to the polyclonal B cell mitogen LPS (Tahir et al., 1993; Faisal et al., 1991b). Suppressed B cell response is demonstrated in a plaque forming cell assay in salmon injected with DMBA and then challenged with T-dependent or T-independent antigen (Arkoosh et al., 1994).

The reported differences between lymphocyte function in fish and mammals due to PAH exposure may be due to several things. There are differences between exposure techniques used in aquatic and mammalian exposures. Both types of trials use exposure by injection, by in vitro incubation with compounds, and through diet. However, it is common in aquatic exposures to expose fish via contaminated sediments in their aquaria, or to directly assay fish from environmentally contaminated settings. These two techniques are not frequently used in mammalian assays. Another difference between mammalian and aquatic PAH exposures is in the compounds used for exposure. In aquatic exposures, interestingly enough, work has been done primarily with mixtures of PAH, such as those found in diesel-based
drilling muds, contaminated harbors, or PAH reference mixtures. In mammalian work, there is little information concerning the effects of PAH mixtures on immune response. Aquatic PAH immunoassays, with a few exceptions, have been done with mixtures. Another difference between mammalian and aquatic assays is that lymphocytes primarily from the head kidney rather than peripheral blood have been used in the teleost assays. This is probably due to the relative abundance of cells in the head kidney as opposed to peripheral blood. In mammalian assays, lymphocytes from a variety of sources are used. By assaying cells from different organs, responses are likely to be different. Another source of variation between aquatic and mammalian assays is the variety of fish species used from a variety of sources. For example, feral fish may be more resistant to toxicant challenge than fish from hatcheries due to the combined effects of factors such as adaptation, development of resistance, and genetic heterogeneity. All of these differences may contribute to the variation between results observed in mammalian and teleost lymphocyte assays.

Of all the sources of variation between mammalian and teleost lymphocyte response, the one that seems to be most important is that fish PAH assays have been done primarily with PAH mixtures. There is little information concerning mammalian response to a mixture of PAH. The effect of a mixture on immune response may be very different from the effect of a single compound. Results in mammalian studies might be different if the murine or human cells were challenged with a mixture of PAH, rather than individual compounds. Exposure to mixtures provides interesting experimental results due to the resemblance to realistic environmental challenges. In
the study presented in this paper, both individual compounds and a defined mixture of PAH are used.

**PAH tested for immunotoxicity**

The following compounds were selected for this study due to their abundance in environmental energy-related waste, and their mutagenic and carcinogenic properties: 2-aminoanthracene (2-AA), 2-methylnaphthalene (2-MN), and 9,10-dimethylanthracene (9,10-DMA) (Fig. 6). Because there is a correlation between carcinogenicity and immunotoxicity, these compounds were chosen as likely immunotoxins. There is currently little in the literature concerning immunotoxicity of 2-AA, 2-MN, and 9,10-DMA. This study identifies these compounds as immunomodulatory agents.

**2-Aminoanthracene**

2-Aminoanthracene (CAS Registry Number 613-13-8) is a three ring PAH with an amine group in the number 2 position. It is classified as a tumorigenic and mutagenic agent by NIOSH. Physical properties of 2-AA include: molecular weight 193.25, melting point 238-241°C, boiling point 93°C, and water solubility, 0.1mg/ml at 22°C. It is a brown powder at room temperature.

**9,10-Dimethylanthracene**

9,10-Dimethylanthracene (CAS Registry Number 781-43-1) is a three ring PAH with methyl groups in the 9 and 10 positions. It is classified by NIOSH as a tumorigen and mutagen. Physical properties of 9,10-DMA include: molecular weight 206.3, melting point 181-183°C, boiling point 360°C, and water solubility 0.056 mg/L. It is a yellow crystal at room temperature.
Fig. 6 2-methylnaphthalene (2-MN), 2-aminoanthracene (2-AA), 9,10-dimethylanthracene (9,10-DMA).
2-Methynaphthalene

2-Methynaphthalene (CAS Registry Number 91-57-6) is a 2 ring PAH with a methyl group in the number 2 position. It is classified by NIOSH as a mutagen. Physical properties of 2-MN include: molecular weight 142.2, boiling point 241.1°C, melting point 34.4°C, and water solubility 24.6-27.3 mg/L at 25°C. It is a white crystal at room temperature.

Objectives

1) To determine the effects of 2-AA, 2-MN, 9,10-DMA, and a mixture of these compounds on bluegill lymphocyte proliferation and intracellular calcium levels.
2) To identify possible mechanisms for PAH effects on bluegill lymphocyte function.
3) To add to the body of knowledge that describes the effects of immunotoxins on signal transduction pathways.

Hypotheses

1) 2-MN, 2-AA, 9,10-DMA, and a mixture of these compounds will be immunomodulatory to bluegill lymphocytes after a 14-day feeding of these compounds in doses previously determined not to cause mortality.
2) The immunomodulatory effects due to PAH-feeding are the result of alterations in lymphocyte signal transduction, and these alterations in signal transduction may be identified by results from mitogen-stimulated proliferative assays and measurements of baseline intracellular calcium.
3) A 14-day PAH-feeding will reduce lymphocyte proliferation and increase baseline intracellular calcium levels.
Chapter 2
Methods and Materials

Fish

*Lepomis macrochirus*, weighing 51-156gm and measuring 13-18cm, were collected from the Louisiana State University Lakes. Fish were acclimated for two weeks in 100 liter glass aquaria, one fish per tank, in dechlorinated water. During the acclimation period, fish were fed trout chow floating pellets (Ziegler, Gardeners, PA). The water was changed daily, and was maintained at 20 - 22° C.

Diets

All polycyclic aromatic hydrocarbons (PAH>95% purity as assessed by the manufacturer), including 9,10-dimethylanthracene, 2-methylnaphthalene, and 2-aminoanthracene were purchased from Sigma Chemical Company (St. Louis, MO). Pellets were prepared by dissolving 2-AA, 2-MN and 9,10-DMA in corn oil (Sigma) and mixing with pulverized trout chow (Ziegler). The diets were homogenized by mixing on a roller mill for three days. Pellets were then formed by forcing the mixture through a 60cc catheter tip syringe into pellets forms (Fisher, Houston, TX). Pellet diets of individual compounds (2-AA, 2-MN, 9,10-DMA) were made at 220μg/pellet for a total 14 day dose of 3.1±.01mg. Each pellet of the mixture of compounds (MIX) contained 134μg 2-AA, 189μg 9,10-DMA, and 222μg 2-MN for a total 14 day dose of 0.13±.01mg 2-AA, 0.19±.01mg 9,10-DMA, and 0.22±0.02mg 2-MN (Table 1). In the 21 day range finding pilot experiment, doses were 0.5, 2.0, and 3.0 mg/kg body weight/day. The vehicle control diet was made up of trout chow and corn oil only. Diets were stored in the dark at 0° C. Fish were fed one pellet per day for 14 days, or

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Table 1 Results from triplicate analysis of pellet diet in milligrams. Each number represents total PAH content of a single pellet.

<table>
<thead>
<tr>
<th>Individual PAH Diet</th>
<th>Mix Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methynaphthalene</td>
<td>0.216</td>
</tr>
<tr>
<td></td>
<td>0.212</td>
</tr>
<tr>
<td></td>
<td>0.232</td>
</tr>
<tr>
<td>2-Aminoanthracene</td>
<td>0.242</td>
</tr>
<tr>
<td></td>
<td>0.212</td>
</tr>
<tr>
<td></td>
<td>0.216</td>
</tr>
<tr>
<td>9,10-Dimethylantracene</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td>0.233</td>
</tr>
<tr>
<td></td>
<td>0.215</td>
</tr>
</tbody>
</table>
in the case of the range finding pilot experiment, one pellet per day for 21 days. Fish had been previously trained to eat a floating pellet diet, and consistently ate their pellet immediately upon being fed. Fish were fed at 9:00 a.m. each day, and observed to ensure their dose was eaten.

For analysis of pellet content, pellets containing either 2-MN or 9,10-DMA were sonicated and extracted with dichloromethane (Mallinckrodt, Paris, KY). Pellets containing 2-AA were extracted with 80% dichloromethane and 20% methanol (Mallinckrodt). Extracts were cleaned up to remove lipids by passing through activated fluorosil (Sigma). The EPA mix of deuterated PAH was used as an internal standard for the samples, and 2-fluorobiphenyl was used as an instrument standard. Samples of 2µl were analyzed using an HP Model 5980 gas chromatograph interfaced to an HP model 5970 quadrupole mass spectrometer.

Cell separation

Lymphocytes were collected from peripheral blood according to Miller (Miller, 1988). Approximately 0.5ml blood was collected from bluegill anesthetized in dechlorinated water containing .25g/l tricane methane sulfonate (Sigma). Blood was collected from the caudal sinus at the base of the anal fin using a 1cc tuberculin syringe and 22 gauge needle containing 100 units heparin sodium (Elkins-Sinn, Cherryhill, NJ). The collected blood was immediately suspended in 2ml of incomplete media (Miller, 1988) in a 5ml polystyrene tube, and underlayered with 1-2ml of Lymphoprep density gradient (Gibco, Grand Island, NY). The blood was spun for 30 minutes at 300g in a Beckman GPKR swinging rotor centrifuge to separate the cells. The cell layer at the Lymphoprep-plasma interface was collected, washed twice
in 10x volume of media to remove any Lymphoprep, and then resuspended in complete medium (containing 5% bluegill serum, 1% L-glutamine). Cells were counted and viability was measured using the trypan blue (Sigma) dye exclusion method. Cell viability was greater than 95%.

**Cell proliferation**

Cell proliferation assays were done in 96-well plates at 500,000 cells per well, 200µl final volume. Cells were cultured in osmotically adjusted (10% tissue culture water) L-15/AIM V (Gibco) media supplemented with 5% bluegill serum (Miller, 1988). Mitogens were added to the cell culture at the time of incubation. Concanavalin A was used at a final concentration of 25µg/ml, phorbol myristate acetate (PMA) was used at a final concentration of 2ng/ml, and calcium ionophore (A23187) was used at a final concentration of 200ng/ml. All mitogens were sterile tissue culture grade and purchased from Sigma. Mitogens were dissolved in sterile tissue culture grade anhydrous dimethyl sulfoxide (DMSO, Sigma) just prior to use. Cells were incubated at 25°C in a 5% CO₂ humidified environment (Forma Scientific, Marietta, OH). PMA and A23187 were removed from the cell culture media 18 hours after initial incubation, and replaced with complete media, due to cytotoxicity after 20 hours. Cells were pulsed after 40 hours of incubation with 0.5µCi [³H] thymidine per well, and harvested 20 hours later. The radioactivity was counted in a liquid scintillation counter (LKB Wallac 1205 Betaplate Liquid Scintillation Counter). Cell proliferation is described as the count of [³H] thymidine incorporation into new DNA.

**Flow cytometry measurement of calcium**

Bluegill lymphocytes from PAH-fed fish and control fish were analyzed for differences in resting or baseline intracellular Ca²⁺. Cell samples were prepared in
triplicate for each treatment and control group. Baseline intracellular Ca\(^{2+}\) levels were analyzed using a FACScan Flow Cytometer (Beckton Dickinson, Bedford, MA) equipped with an argon ion (488nm emission) laser. The Ca\(^{2+}\)-chelating dyes, Fluo-3 AM and Fura-Red AM (Molecular Probes, Eugene, OR) were used as a ratiometric indicators of Ca\(^{2+}\). Fura-Red fluoresces most intensely in the red region when not bound to Ca\(^{2+}\), and Fluo-3 fluoresces with increasing intensity in the green region when bound to Ca\(^{2+}\). By loading the two dyes simultaneously, and using a ratiometric approach, heterogeneity in dye loading becomes less important, and the magnitude of response is greater than achieved using only one dye. These dyes used simultaneously give a low noise, highly sensitive measurement of free intracellular Ca\(^{2+}\) (Novak and Rabinovich, 1994). Lymphocyte samples were suspended at 500,000 cells/100μl media containing 6μM Fluo-3 and 13.8μM Fura-Red. Cell viability was assessed as 95-98% by trypan blue (Sigma). Cells were incubated for 1.5 hours at 25°C in a 5%CO\(_2\) environment, with cells being shaken at 45 minutes to ensure even loading. Just prior to analysis, media volume was brought up to 800μl. Lymphocyte populations were gated on the flow cytometer from monocytes and debris on the basis of forward vs. right angle scatter. The dyes were excited at 488 nm with Fluo-3 emission detected at 515-535nm and Fura Red emission detected at 665-685nm. 10,000 events were counted for each sample. Baseline cellular response was determined using DMSO control treated cells. Addition of 1 μM thapsigargin (Sigma), a specific sarcoplastic/endoplasmic reticulum calcium ATPase inhibitor, served as a positive control for Ca\(^{2+}\) mobilization. Results were analyzed using Verity Winlist software, and were generated as a ratio of
mean channel fluorescence Fluo-3/Fura-Red x 500. The means of these ratios are reported.

**Experimental design**

Fish were randomly assigned to numbered 100 liter tanks, one fish per tank. Each fish was randomly assigned to a feeding schedule of one of the following: 2-AA, 2-MN, 9,10-DMA, or a mixture of the three (MIX). Sample sizes were n=3 to n=5. Feedings were done once a day for 14 days at 221.4μg/day for the individual compounds. The mixture doses were as follows: 134.2μg/day 2-AA, 189.3μg/day 9,10-DMA, and 222.5μg/day 2-MN.

**Statistical analysis**

Data were analyzed for normality, significant differences and interactions. A mixed function model that incorporated the variables diet, mitogen, and a significant diet*mitogen interaction was used. Means were calculated for each triplicate analysis, and the log of the mean was used for the analytical procedure. Means rather than raw data were used due to non-normally distributed residuals of the raw data. Values were considered significantly different at a $p$ value < 0.02. SAS statistical software was used to write the program for data analysis.
Chapter 3
Results

21-day range finding pilot experiment

Dose and length of feeding effects on cell proliferation

Fish were fed a dose of 0.5, 2, or 3 mg/kg body weight/day of one of the following: 2-MN, 2-AA, 9,10-DMA, or MIX. Peripheral blood samples were taken at 3, 5, 10, and 21 days. There was no consistent relationship between change in dose and change in proliferation (Figs. 7-9). This lack of relationship may be due to one of the following: 1) small sample size (1-2 fish per dose) or 2) small range in dosing (39-194μg/pellet). Because there is a five fold difference between the smallest and the largest dose, the lack of correlation is likely due to the small sample size. We would expect the dose and rate of proliferation to be related in a linear or quadratic fashion in a larger sample.

Mitogen-stimulated proliferative values are greater at 21 days of feeding than at 3 days of feeding (Fig. 10). However, unstimulated cells from PAH-fed fish demonstrate suppressed proliferation when compared to controls (Fig 11-13).

Effect of compound on cell proliferation

A 21-day feeding of 2-MN, 9,10-DMA, and 2-AA all increased mitogen-stimulated cellular proliferation as compared to controls. However, only 2-AA affected proliferation significantly (Fig. 14).

14-day PAH feeding studies

Lymphocyte proliferation in the absence of mitogen

Lymphocytes from fish fed the diet of 2-aminoanthracene proliferated in the absence of any mitogen. The 2-AA proliferative response was approximately five
Fig. 7 Mitogen stimulated proliferation in bluegill lymphocytes after a 21-day feeding of 2-aminoanthracene. Mitogen: 5μg Con A/500,000 cells.
Fig. 8 Mitogen stimulated proliferation in bluegill lymphocytes after a 21-day feeding of 9,10-dimethylnaphthalene. Mitogen: 5μg Con A/500,000 cells.
Fig. 9 Mitogen stimulated proliferation in bluegill lymphocytes after a 21-day feeding of 2-methylnaphthalene. Mitogen: 5µg Con A/500,000 cells.
<table>
<thead>
<tr>
<th>Concentration</th>
<th>3 Days</th>
<th>21 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>.5 ppm</td>
<td>11,370</td>
<td>47,674</td>
</tr>
<tr>
<td>2 ppm</td>
<td>17,155</td>
<td>50,489</td>
</tr>
<tr>
<td>3 ppm</td>
<td>7,693</td>
<td>26,760</td>
</tr>
</tbody>
</table>

Fig. 10 Proliferation in cells from fish fed 2-aminoanthracene: representative data. Mitogen: 5μg Con A. Response: counts/minute.
Fig. 11  Proliferation of unstimulated lymphocytes: cells from fish fed a 21-day diet of 9,10-dimethylnaphthalene vs. control.

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Fig. 12 Proliferation of unstimulated lymphocytes: cells from fish fed a 21-day diet of 2-methylnaphthalene vs. control.

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Fig. 13 Proliferation of unstimulated lymphocytes: cells from fish fed a 21-day diet of 2-aminoanthracene vs. control.
Fig. 14 Mitogen stimulated lymphocyte proliferation measured after 21-day PAH feeding. CPM = log transformed mean counts/minute. PPM = parts per million dose. Mitogen stimulation: 5µg Con A/500,000 cells.
times that of the control (Fig. 15). However, the MIX diet, which contained 2-AA had reduced proliferative response as compared to controls (Fig. 16). The proliferative response of cells from the 2-MN and 9,10-DMA treated cells was reduced as compared to the control, but not significantly. Counts per minute (CPM) of the control ranged from 200 to 1700.

**Con A induced proliferative responses**

Proliferative response to the T cell mitogen, Con A was significantly reduced in lymphocytes from fish fed 2-MN, and 9,10-DMA. Lymphocytes from fish fed 2-AA demonstrated proliferation greater than the control in response to treatment with Con A, although not significantly so (Fig. 17). The CPM range for the control was 5000 to 25,000. The concentration of Con A used in this assay was 25μg/ml. This concentration was previously determined in a pilot experiment to be an optimal mitogen dose.

**Phorbol ester (PMA) stimulated lymphocyte proliferation**

PMA does not stimulate cell proliferation in the absence of monocytes or exogenous addition of IL-2 (Hashimoto *et al.*, 1991). However, Ficoll density gradient separated cells will often proliferate with the addition of only PMA (Hashimoto *et al.*, 1991), due to the presence of monocytes. Catfish lymphocytes are not stimulated with phorbol ester alone (Lin *et al.*, 1992), although red drum lymphocytes have shown a stimulation response (Burnett *et al.*, 1994). Bluegill control lymphocytes did not respond to PMA administration (200-2,000 CPM), but the cells from the 2-MN treated fish and the 2-AA treated fish (Fig. 18) demonstrated significant proliferation (*p*<0.02). The CPM for 2-AA ranged from 6,000 to 15,000.
Fig. 15 Lymphocyte proliferation in the absence of mitogen stimulation. 14-day PAH total dose/fish: 3.1±0.1 mg. Values are means ± SEM. Values significantly different * from those of vehicle control at p<.02.
Fig. 16 Mitogen effect on lymphocyte proliferation from fish fed 14-day diet of MIX vs. vehicle corn oil control. Concentrations in cell culture: Con A 25mg/ml, PMA 2ng/ml, A23187 200ng/ml, PMA/A23187 202ng/ml. PAH total dose/fish: 0.13 ± 0.01 mg 2-AA, 0.19 ± 0.01 mg 9,10-DMA, and 0.22 ± 0.02 mg 2-MN. Values are means ± SEM. Value significantly different * from vehicle control at p<.02. None= media only. ConA= concanavalin A. PMA= phorbol myristate acetate. A23187= calcium ionophore. CPM= counts per minute.
Fig. 17 Concanavalin A-stimulated lymphocyte proliferation. Con A concentration in cell culture: 25mg/ml. 14-day PAH total dose/fish: 3.1 ± .01 mg. Values are means ± SEM. Values significantly different * from those of vehicle control at p<.02.
Fig. 18 Phorbol ester-stimulated lymphocyte proliferation. Phorbol ester concentration in cell culture: 2ng/ml. 14-day PAH total dose/fish: 3.1 ± .01mg. Values are means ± SEM. Values significantly different * from those of vehicle control at p<.02.
and the CPM for 2-MN ranged from 4,000 to 7,000. Lymphocytes from fish fed the 9,10-DMA diet demonstrated proliferation in the same range as the control.

**Calcium ionophore stimulated lymphocyte proliferation**

Calcium ionophore does not stimulate cell proliferation in the absence of monocytes or addition of exogenous IL-2 (Hashimoto *et al.*, 1991). In this study, bluegill control cells did not respond to calcium ionophore alone (Fig. 19). The proliferation due to calcium ionophore was significantly increased relative to controls in cells from 2-MN treated fish (4,000-12,000 CPM) and from 2-AA treated fish (6,000 to 17,000). Control cells CPM ranged from (500 to 4,000). Cells from 9,10-DMA treated fish were stimulated less than the control, but not significantly so.

**Calcium ionophore/phorbol ester stimulated lymphocyte proliferation**

The greatest proliferation was demonstrated in bluegill cells treated with a combination of calcium ionophore and phorbol ester. PMA/A23187 stimulated greater proliferative counts than Con A, due to the fact that Con A is a T cell mitogen, and calcium ionophore/phorbol ester stimulates both T and B cells (Clevers, 1985). Proliferation of the control ranged from 4,000-21,000 CPM (Fig. 20). Significantly less lymphocyte proliferation was observed in 9,10-DMA-treated fish (300-3000 CPM). Cells from 2-MN treated fish proliferated approximately equally to the control, and cells from 2-AA treated fish proliferated in the range of 5,000-12,000 CPM. The 2-AA response to the combination of calcium ionophore/phorbol ester was suppressed as compared to controls, and was less than the proliferation stimulated in 2-AA cells by either phorbol ester or calcium ionophore alone.

**Effects of 2-MN, 2-AA, and 9,10-DMA diets on lymphocyte proliferation**

Lymphocytes from 9,10-diethylanthracene-fed fish demonstrated significantly reduced lymphocyte proliferation in response to all mitogens (Fig. 21).
Fig. 19 Calcium ionophore-stimulated lymphocyte proliferation. Calcium ionophore concentration in cell culture: 200ng/ml. 14-day PAH total dose/fish: 3.1 ± .01 mg. Values are means ± SEM. Values significantly different * from those of vehicle control at \( p < .02 \).
Fig. 20 Lymphocyte proliferation as stimulated by calcium ionophore and phorbol ester. A23187/PMA concentration in cell culture: 202ng/ml. 14-day PAH total dose/fish 3.1 ± .01 mg. Values are means ± SEM. Values significantly different * from those of vehicle control at p<.02.
Fig. 21 Mitogen effect on lymphocytes from fish fed 14-day diet of 9,10-dimethylanthracene vs. vehicle corn oil control. Concentrations in cell culture: Con A 25μg/ml, PMA 2ng/ml, A23187 200ng/ml, PMA/A23187 202ng/ml. 9,10-DMA total dose/fish 3.1±0.01mg. Values are means ± SEM. Value significantly different * from vehicle control at p<.02. None= media only. ConA= concanavalin A. PMA= phorbol myristate acetate. A23187= calcium ionophore. CPM= counts per minute.
The effect of the 2-methylnaphthalene diet on lymphocyte proliferation was variable depending on the mitogen used (Fig. 22). Response of cells from fish fed 2-MN was depressed compared to the control in the absence of mitogen and in the presence of Con A. The diet of 2-aminoanthracene had a mitogenic effect on cell proliferation (Fig. 23).

Effects of the MIX diet on lymphocyte proliferation

The MIX diet cannot actually be compared to the other diets due to a difference in diet concentrations. The MIX diet was a total dose of $7.56 \pm 0.56$ mg PAH over the 14 days, whereas the individual diets were $3.1 \pm 0.01$ mg total PAH dose over 14 days. However, the MIX diet suppressed proliferation in all response to all mitogens used (Fig. 16), and the average level of MIX proliferation was approximately equal to proliferation of control cells in the absence of any mitogen.

Baseline intracellular calcium in lymphocytes from PAH-fed fish

Resting, or baseline intracellular calcium in cells from PAH-fed fish was slightly reduced as compared to cells from control fish (Fig. 24). The lowest intracellular calcium was demonstrated in cells from fish fed 2-MN and 2-AA, which correlates with proliferative changes due to these diets. Baseline calcium in cells from the MIX diet was reduced, however calcium levels in cells from fish fed 9,10-DMA were similar to control values. Results are depicted as mean channel fluorescence of green/red x 500 (Fig. 25). The mean value for the control is approximately 70. Mean values for 2-AA and 2-MN treated fish are approximately 56.

Cell counts resulting from 21-day and 14-day PAH feedings

There was a greater number of lymphocytes in the blood from the fish fed a 14-day diet of PAH as compared to a 21-day diet of PAH (Table 2). However, the viability of the cells present was 95-100% in both cases.
Fig. 22 Mitogen effect on lymphocytes from fish fed 14-day diet of 2-methynaphthalene vs. vehicle corn oil control. Concentrations in cell culture: Con A 25μg/ml, PMA 2ng/ml, A23187 200ng/ml, PMA/A23187 202ng/ml. 2-MN total dose/fish 3.1±.01mg. Values are means ± SEM. Value significantly different * from vehicle control at p<.02. None= media only. ConA= concanavalin A. PMA= phorbol myristate acetate. A23187= calcium ionophore. CPM= counts per minute.
Fig. 23 Mitogen effect on lymphocytes from fish fed 14-day diet of 2-AA vs. vehicle corn oil control. Concentrations in cell culture: Con A 25μg/ml, PMA 2ng/ml, A23187 200ng/ml, PMA/A23187 202ng/ml. 2-AA total dose/fish 3.1±.01mg. Values are means ± SEM. Values significantly different * from vehicle control at p<.02. None= media only. ConA= concanavalin A. PMA= phorbol myristate acetate. A23187= calcium ionophore. CPM= counts per minute.
Fig. 24 Baseline intracellular calcium level in *Lepomis* lymphocytes after 14-day PAH feeding. Cells were incubated with FLUO-3 and Fura Red. Results are shown as ratio mean channel fluorescence (FLUO-3/Fura Red)x500. Values are means ± SEM. Cells from PAH-fed fish show decreased baseline Ca^{2+} compared to DMSO solvent control.
Fig. 25 Example of flow cytometry baseline intracellular Ca\textsuperscript{2+} levels. Y axis represents number of cells per channel, X axis represents ratio mean channel fluorescence. Representative ratio histograms from A) fish eating control diet for 14 days and B) fish eating 3.1±.01 mg 2-AA total dose for 14 days.

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Table 2 Representative data of viable cell counts after 21-day and 14-day feedings of 2 mg/kg/day of individual PAH compounds. MIX dose is 7 mg/kg/day. Cell count listed is median count in each case.

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<tr>
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<th>14-Day Feeding</th>
<th>21-Day Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-MN</td>
<td>$2.2 \times 10^7$</td>
<td>$1.2 \times 10^7$</td>
</tr>
<tr>
<td>2-AA</td>
<td>$1.76 \times 10^7$</td>
<td>$7.1 \times 10^6$</td>
</tr>
<tr>
<td>9,10-DMA</td>
<td>$2.9 \times 10^7$</td>
<td>$5.9 \times 10^6$</td>
</tr>
<tr>
<td>MIX</td>
<td>$1.3 \times 10^7$</td>
<td>$0.1 \times 10^6$</td>
</tr>
<tr>
<td>Control</td>
<td>$1.75 \times 10^7$</td>
<td>$1.3 \times 10^7$</td>
</tr>
</tbody>
</table>
Chapter 4
Discussion

Carcinogenic PAH have been demonstrated to have a suppressive effect on lymphocyte proliferation in humans and mice (for review see Davila et al., 1995), and highly variable effects on lymphocyte proliferation in fish (Faisal et al., 1991; Faisal and Huggett, 1993; Arkoosh et al., 1996; Tahir et al., 1995). There is a strong correlation between carcinogenicity and immunosuppression (White and Holsapple, 1984). BaP and DMBA are two well characterized carcinogenic PAH that are also immunosuppressive (Dean and Adams, 1985; White, 1986) in mammals. In the present research, mutagenic PAH were tested for their immunomodulatory characteristics using the lymphocyte proliferation assay and a number of mitogens which interact at various places in cell signaling pathways. Three compounds were tested for immune effects individually and as a mixture: 2-MN, 9,10-DMA, and 2-AA.

9,10-Dimethylanthracene and the mixture of compounds (MIX) were immunosuppressive. Lymphocytes from fish fed these two PAH diets exhibited significantly reduced lymphocyte proliferation in response to all mitogens: Con A, PMA, A23187, and PMA+A23187. Suppression of immune function after exposure to 9,10-dimethylanthracene has been demonstrated previously in human T cells (Davila et al., 1996). Therefore, the response of immunosuppression in bluegill due to 9,10-DMA is consistent with responses observed in higher vertebrates. The response of cells to the MIX diet has not been investigated previously and was therefore not predictable. Lymphocyte response to a mixture of PAH has not previously been studied in human and murine models. In fish, environmental mixtures of PAH
compounds have been demonstrated to have varying effects on lymphocyte proliferation. There are reports of immunosuppression in response to a mixture of PAH and reports of enhanced lymphocyte proliferation (Faisal et al., 1993; Faisal et al., 1991; Tahir et al., 1993; Tahir and Secombes, 1995; Arkoosh, 1994; Arkoosh, 1996). Although our study demonstrates significant immune suppression as a result of the MIX diet, there are other reports of proliferation due to exposure to PAH mixtures in fish. In these reports, the fish have been collected from an environmentally contaminated setting (Faisal et al., 1991; Arkoosh et al., 1996). It has been suggested that this proliferative response is actually the response of carcinogen-resistant cells proliferating selectively relative to surrounding cells, and that the initial effect of the PAH carcinogens is actually inhibitory. The later proliferative response is considered clonal adaptation in response to the PAH challenge (Farber, 1990). In our study, we demonstrated suppression of proliferation due to a dietary exposure to 9,10-DMA and the MIX diet, rather than proliferation as seen in some environmentally contaminated settings. It is possible that in addition to clonal adaptation of feral fish lymphocytes, there is the additional mechanism of route of exposure affecting the action of PAH on fish immunity. In our study the route of exposure is dietary, and metabolic action is via the gut and liver. The primary route of exposure for the environmentally exposed fish is likely to be via the gills. The two routes of exposure may have different rates and mechanisms of metabolism, resulting in different effects on lymphocyte proliferation.

It is also interesting to note that the MIX diet is suppressive to lymphocyte proliferation even though it contains 2-AA and 2-MN which, alone, act as stimulants.
to proliferation. The interaction of 2-AA, 2-MN, and 9,10-DMA in the MIX diet somehow functions to suppress proliferation in all instances, whereas the individual compounds induce proliferation in some cases. The mechanisms involved in suppression of activation by a mixture such as this are not clear, but warrant further investigation.

The effect of the 2-methylnaphthalene diet on lymphocyte proliferation was variable depending upon the mitogen used. Response of cells from fish fed 2-MN was depressed compared to the control in the absence of mitogen and in the presence of Con A. This response is not surprising in general due to previous reports of PAH depression of Con A stimulated cell proliferation (Yamashita and Hamoaka, 1982a; Thurmond et al., 1988). The effect of 2-MN on cell proliferation stimulated by calcium ionophore, phorbol ester, and a combination of the two is a novel response that has not been previously observed. In these instances, the diet of 2-MN enhanced the proliferative response relative to controls. This is interesting in light of the overall effect of 2-MN on cell activation being suppressive as evidenced by the reduced response to Con A. 2-MN may mediate alteration of signal transduction in such a way that the overall effect is suppression. Because the 2-MN treated cells responded to calcium ionophore and phorbol ester, it seems that the 2-MN effect on the PKC/ras and calcium pathways was actually stimulatory. It is possible that 2-MN may exert effects on various components of the signal transduction pathway, with the overall effect being suppression. However, there may be effects that 2-MN exerts that are actually stimulatory, but are not apparent until the addition of phorbol ester to stimulate ras/PKC and the addition of calcium ionophore to stimulate the calcium
pathway. 2-MN may in some manner trigger a small amount of calcium mobilization and ras signaling, which alone is not sufficient to generate proliferation. However, when given the additional signal of phorbol ester or calcium ionophore, the synergy is sufficient to stimulate proliferation.

2-MN at a dose of 42mg/kg has been previously demonstrated to stimulate proliferation of bronchial epithelial cells in Swiss-Webster mice 72 hours after I.P. injection, and suppression of proliferation 14 days after injection (Rasmussen et al., 1986). This variation in proliferative response over a period of time suggests that metabolic activation is involved in controlling the 2-MN proliferative response. In our study, a comparable dose of approximately 39mg/kg total PAH over a 14 day period generated an overall response of suppression, with stimulatory effects on certain pathways. Glutathione, whose role is to buffer the effects of reactant oxidants on sulfhydryl oxidation, has been shown to be reduced in DBA/2J mice as a result of 2-MN exposure (Griffin et al., 1982). Reactive metabolites of 2-MN (dihydodiol) have been shown to bind irreversibly to macromolecules in mice (Griffin et al., 1981; Griffin et al., 1982). It is possible that the reactive metabolites of 2-MN are binding and activating sensitive proteins in the PKC, ras, or calcium pathways. This type of reactive metabolite signaling has been demonstrated with other compounds such as nitric oxide, HgCl₂, and H₂O₂ (Lander, 1997). The inhibitory effects of 2-MN as evidenced by suppressed Con A stimulation may be due to 2-MN metabolites having an effect on later stages of signal transduction. The inhibitory effects may be acting on transcription factors, or even late stages of the ras pathway, such as MAP kinase. When PMA or calcium ionophore is added to the cell culture, the stimulatory effects
on PKC, ras and calcium may be sufficient to overcome the inhibition in the later stages of the pathway.

Another possible explanation for the suppressive effects of 2-MN on Con A induced proliferation, but stimulatory effects in response to A23187 and PMA, is inhibition of early tyrosine phosphorylation events. If this is the case, the inhibition of tyrosine phosphorylation would account for the lack of response to Con A. By inhibiting early tyrosine phosphorylation events that are essential to all other cell activation events (June et al., 1990), 2-MN could demonstrate reduced response to Con A stimulation. The stimulatory effect of PMA and A23187 on PKC, ras, and calcium pathways could be explained as 2-MN metabolites directly activating proteins in those pathways, bypassing tyrosine phosphorylation. However, this stimulation of proteins may be small, and only be revealed upon synergistic action with exogenous stimulants (PMA, A23187). A23187/PMA direct activation of proteins and cellular response bypassing tyrosine phosphorylation events, has been demonstrated in many models (Clevers et al., 1985).

To summarize, the following possible explanations for the effects of 2-MN on cell proliferation, mediated by signal transduction, are offered: The metabolites of 2-MN may play a role in altering cell proliferation/suppression. Reactive 2-MN metabolites, possibly dihydrodiols, may bind and activate, or bind and inhibit certain sulfhydryl-sensitive signaling proteins. In particular, the metabolites may activate in a small way, components of the ras, PKC or calcium pathways in such a way that proliferation is not induced, but that the addition of exogenous stimulants such as PMA or calcium ionophore can synergize to cause proliferation. The inhibitory
effects of 2-MN metabolites as evidenced by Con A suppression, may be on late stages of ras activation such as activation of MAP kinase or DNA transcription factors. This would explain the overall suppressive effect of 2-MN. The activation of PKC, ras, and calcium by PMA and A23187, may be sufficient to overcome the 2-MN inhibition of late stage signaling, as evidenced by the proliferation induced by phorbol ester and calcium ionophore. Alternately, the inhibitory effects of 2-MN or its metabolites may be on early stages of tyrosine phosphorylation. This would explain suppression of Con A induced proliferation, and allow for PMA/A23187 enhanced proliferation. With this explanation, 2-MN could directly activate PKC, ras, and calcium signaling proteins in a small way. These effects would be revealed by the synergistic actions of A23187 and PMA, which bypass early tyrosine phosphorylation events.

The promutagenic aromatic amine, 2-aminoanthracene does not exhibit a suppressive effect on lymphocyte proliferation. Instead, it has a mitogenic or stimulatory effect on cell proliferation itself. Other aromatic amines have been shown to stimulate cell proliferation. Lee et al (1996) reported enhanced proliferative response in murine splenocytes to N-hydroxy-2aminofluorene, a primary metabolic product of 2-aminofluorene. 2,4-diaminotoluene has been demonstrated to produce a strong proliferative response in C57Bl/6 hepatocytes from mice fed a 10-day diet of this carcinogen (Suter et al., 1996).

A possible route of activation for these aromatic amines is N-hydroxylation and then conversion to reactive nitrenium ions. 2-Aminoanthracene can be bioactivated by P450 enzymes to N-hydroxy-2-aminoanthracene. The hydroxyamine
can be O-acetylated, and then further activated to a reactive nitrenium ion. Deactivation routes include N-acetylation and conjugation with glutathione (Parkenson, 1996). It is possible that proliferative responses resulting from exposure to aromatic amines are in some way the result of the metabolic activation of the amine group, rather than oxidation of the aromatic ring as in 9,10-DMA.

2-Aminoanthracene stimulates cellular proliferation in the absence of any additional mitogen. This response is surprising when compared to other mutagenic PAH. Commonly, carcinogenic PAH suppress immune response as measured by the lymphocyte proliferation assay (Farber, 1976, Farber, 1990). Because 2-aminoanthracene stimulates proliferation rather than suppresses it, it is likely that an alternate signaling pathway is transduced in response to this aromatic amine.

2-Aminoanthracene undergoes P450 activation, like 2-aminofluorene, to an N-hydroxyamine (Fig. 26). The hydroxyamine can be O-acetylated, and subsequently converted to a reactive electrophile (nitrenium ion) that covalently binds DNA in the nucleus, ultimately leading to mutational events. O-acetylation and deacetylation of aromatic amines, resulting in a reactive nitrenium species, is a major route of metabolic activation for these compounds. One possibility is that the reactive metabolite of 2-AA could bind the DNA in a fashion mimicking transcription factors and thus stimulate IL-2 transcription. Stimulation of the IL-2 gene requires the coordinated action of several transcription factors. If the reactive metabolite of 2-aminoanthracene could bind the IL-2 gene promoter region in enough locations to emulate transcription binding, the gene could possibly be activated. This model would
Fig. 26 The role of N-acetyltransferase in the O-acetylation of 2-aminoanthracene.
account for proliferation in the absence of a mitogen, and the metabolite would act as
the mitogen itself.

2-aminoanthracene or a metabolite may serve to activate or stimulate the IL-2
receptor (IL-2R). There are instances of aromatic amines being able to bind to such
receptors. Aromatic amines have been shown to have some binding affinity for
estrogen receptors. The aromatic amine, 2-acetylaminofluorene (2-AF) has been
shown to bind an estrogen-binding protein in the rat liver. 2-AF, which is very similar
in structure to 2-AA, does not bind the TCR (Lee et al., 1996), so possibly the effect
of 2-AA is on the IL-2R or other growth factor receptor. This could be an explanation
for 2-AA induced proliferation in the absence of mitogen. Antigen-induced T cell
proliferation is regulated primarily by the actions of IL-2 on its cell surface receptor
(Weiss, 1993). IL-2 is produced and can act on itself by interacting with its receptor,
thus driving the lymphocyte through the cell cycle. Therefore, stimulation of the IL-
2R by 2-AA or a metabolite of 2-AA could be sufficient to stimulate cell proliferation.

Some compounds, such as mercury, have been shown to stimulate a type of
redox cell signaling by reacting with thiol sensitive signaling proteins. HgCl₂ has
been demonstrated to aggregate cell surface receptors on murine thymocyte and spleen
cells by forming sulfur-mercury bonds (Nakashima et al., 1994). This receptor
aggregation is associated with increased tyrosine phosphorylation and excessive
lymphocyte proliferation. This type of redox signaling is also a possibility for the
reactive electrophiles generated by activation of aromatic amines. Aromatic amines
can be activated via N-hydroxylation, and subsequent N-sulfation (Fig. 27) or by O-
acetylation/deacetylation to reactive electrophiles capable of binding DNA and
Fig. 27 The role of sulfation in the activation of 2-aminoanthracene.
proteins in the rat liver (Miller and Miller, 1981). If these metabolic pathways exist in fish, this type of activation is a possible route for reactive metabolites of 2-AA to bind and stimulate signaling proteins and receptors. Likely candidates for this type of attack would be sulfhydryl sensitive protein tyrosine kinases and phosphatases. Romero (1997) demonstrated depletion of glutathione in response to BaP metabolites in human T cells, and suggests that there is a possibility of BaP metabolite attack on protein sulfhydryl groups in this system. In this case, the result was not cell stimulation, but rather depression of proliferation. It is possible that the binding of reactive intermediates to protein thiols could result in an increase or decrease of signal transduction, depending on the proteins affected.

Several xenobiotic compounds have been demonstrated to chemically induce protein activation: chromium in H4-II-E rat hepatocytes (Kim and Yurkow, 1996) hemin and H$_2$O$_2$ in the human T cell line Jurkat (Lander et al., 1995; Whisler, 1995), and diamide in human neutrophils (Fialkow, 1994). These compounds have in common that they are all oxidizing agents.

Free radicals and redox stress may play a role in mediating signal transduction (Staal et al., 1994). Cells have protective mechanisms to guard against excessive oxidative stress due to free radical generation. In human peripheral blood mononuclear cells, glutathione acts to buffer the effects of reactive oxidants on sulfhydryl oxidation (Reed, 1990). When this protective system is overwhelmed, glutathione is reduced and excess thiol oxidation occurs (Nicotera et al., 1992). Recently, free radicals have been identified as a type of second messenger (Lander, 1997), capable of stimulating signaling by affecting the sulfhydryl sensitive proteins.
which control signaling pathways. Reactive oxygen and reactive nitrogen (Lander et al., 1993b) have been demonstrated to mediate signaling of the transcription factor, NFκB. The iron containing compound, hemin, has been demonstrated to have mitogenic effects on lymphocytes, with free radical generation as a suggested mechanism of activation (Lander et al., 1993a). HgCl₂ is also mitogenic in lymphocytes, possibly through protein thiol binding. Nitric oxide can directly activate ras in vitro by interacting with an important Cys residue in the ras protein (Lander et al., 1995). Also, hemin, H₂O₂, and HgCl₂ are able to directly activate ras in vitro in Jurkat T cells (Lander et al., 1995). The ras pathway is also indicated as a target of thiol-oxidizing free radical signaling by Kim and Yurkow (1996), who demonstrates that chromium activates MAP kinase in a PKC-independent fashion in rat hepatocyte cells.

Free radicals have been shown to play important roles in carcinogenesis by directly damaging DNA (Ames et al., 1993). It is possible that in addition to causing chemical lesions in DNA as a mechanism in carcinogenesis, free radicals may also exert damaging effects on cellular signaling proteins. Reactive nitrenium ions have been shown not only to bind DNA, but also proteins. Clozapine can be oxidized to a reactive nitrenium ion that covalently binds neutrophils and glutathione (Liu and Uetrecht, 1995). The aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), found in fried meats, can be N-oxidized to a reactive species that covalently binds glutathione or thiols (Alexander et al., 1997). The aromatic amine used in this study, 2-aminoanthracene, may undergo activation to a nitrogen centered radical capable of binding DNA and also proteins. If this is the case, the reactive
nitrogen radical, the nitrenium ion, may be able to induce effects in the cell in a similar fashion to other oxidizing agents. The reactive metabolite of 2-AA may stimulate activation of signaling proteins by binding and activating them.

If it is assumed that 2-AA and the other PAH used in this study act directly on signaling proteins, via their reactive metabolic products, then perhaps these actions may be inhibitory rather than stimulatory in some cases. The oxidizing agent, N-ethylmaleimide, oxidizes specific thiols in human lymphocytes resulting in down regulated PLCγ1 and and PTK activity (Kanner et al., 1992). DMBA has been demonstrated to impair HPB-All human T cell function by activating tyrosine kinases Fyn and Lck, possibly causing a detrimental increase in intracellular calcium (Archuleta et al., 1993). A stimulatory action on signaling proteins that results in suppression of function would explain the reduced proliferation in cells from the fish fed 2-MN, 9,10-DMA, and the MIX diet.

All 14-day PAH diets resulted in cells with slightly reduced baseline intracellular calcium as compared to controls. Previous PAH studies have demonstrated varied results concerning elevation or reduction in resting baseline calcium levels. In a similar 14-day feeding study with B6C3F1 mice, a 42mg/kg total dose of DMBA resulted in slightly elevated baseline calcium in lymphocytes from the spleen and peyer’s patch, but reduced baseline calcium in thymocytes (Burchiel, 1992). We demonstrated slightly reduced baseline calcium in peripheral blood lymphocytes after a comparable 39mg/kg total 14 day PAH dose (2-MN, 2-AA, or MIX). The differences in baseline calcium response between our fish study and the mouse study may be explained by different rates and routes of metabolism in the
different organs from which the lymphocytes were collected, if metabolism is indeed involved in this process. *In vitro* incubation with DMBA (direct cell:compound contact), results in elevated baseline calcium in all lymphocytes from B63CF1 mice and in the Jurkat human T cell line, as opposed to oral exposure, which results in some reduced baseline calcium concentrations and some elevated (Burchiel et al., 1992; Burchiel et al., 1990).

Other *in vitro* incubation studies have demonstrated sustained, elevated intracellular calcium levels in response to immunosuppressive PAH, with a correlation between strength of immunosuppression and ability to generate a sustained increase in intracellular calcium concentration (Davila et al., 1995). Interestingly, the metabolites of BaP generate a more sustained elevation of calcium than does the parent compound, indicating a role for P450 metabolism in this process. PAH have also been demonstrated to reduce calcium mobilization and cell proliferation in response to PHA (Davila et al., 1995). The relationship between decreased calcium mobilization and decreased lymphocyte proliferation is straightforward: calcium mobilization in response to ligand binding is necessary to the activation of lymphocytes, therefore reduced calcium mobilization should result in reduced cell proliferation.

Alterations in intracellular calcium levels have also been linked to the induction of oncasis and/or apoptosis. There is a body of information linking increased intracellular calcium levels to the initiation of apoptosis (Bellomo et al., 1992), but there is also a growing body of evidence that demonstrates just the opposite (Baffy et al., 1993; Magnelli et al., 1993; Preston et al., 1997). Reduced intracellular calcium levels have been shown to precede cell death. In the lymphocyte, most of the
calcium is sequestered in the endoplasmic reticulum, and is released into the cytosol after messages from IP$_3$. The emptying of calcium stores into the cytosol, without an accompanying influx of extracellular calcium, as is the case in apoptosis, results in reduced intracellular calcium. The combination of emptying calcium stores and reduced extracellular calcium influx through the plasma membrane has been linked to both oncosis and apoptotic cell death. In this model, the defect is in the plasma membrane and its ability to support capacitive calcium entry. It has been suggested in the macrophage model, that prolonged depletion of cytosolic Ca$^{2+}$ causes irreversible alterations to the plasma membrane (Van Rooijen, 1991).

In this study, our demonstration of reduced baseline calcium may indeed precede cell death. In our pilot studies, 21-day feedings (as compared to 14-day feedings) reduced the overall number of viable cells available for study. Possibly the extra exposure time was enough for cell death to occur. It is interesting that the cells from fish fed 2-MN and 2-AA demonstrated the lowest levels of intracellular calcium. These cells were more effective in stimulating proliferation than the cells from fish fed MIX and 9,10-DMA diets.

The baseline intracellular calcium level comparisons from this study are reported as ratios of mean channel green to red fluorescence multiplied by 500. This is a typical format for presentation of this type of data (Burchiel et al., 1992; Burchiel et al., 1991). The differences in mean channel ratios presented in this study are relatively small, but represent significant changes in calcium concentration and are comparable to ratio changes reported in the available literature. The range of the differences of the means of the transformed data (ratio x 500) presented in this study.
falls within 14 unitless numbers, which is a decrease in intracellular calcium of approximately 22%. The comparable range presented in the study by Burchiel (1991) is 11 unitless numbers, representing a 52% change in intracellular calcium. These changes in calcium concentration may be indicative of approaching cell death.

The mechanisms involved in reduction of cytosolic baseline calcium by the PAH in this study are not clear. It is possible that there is some damage of the plasma membrane, resulting in reduced function for influx of extracellular calcium. The demonstration of reduced calcium as a result of PAH exposure in this research is in agreement with several recent reports (Baffy et al., 1993; Magnelli et al., 1993; Preston et al., 1997), and may be an indication of approaching cell death.

2-AA, 2-MN, 9,10-DMA, and a mixture of these compounds have been demonstrated in this study to be immunomodulatory to bluegill lymphocytes. These PAH alter proliferative response and reduce intracellular baseline calcium levels as compared to controls. The immunomodulatory effects of these PAH may be due to alterations in signal transduction pathways. Further research in this area is necessary to identify specific pathways affected.
Chapter 5
Conclusions

The first hypothesis tested in this study was that a 14-day diet of 2-MN, 2-AA, 9,10-DMA, and a mixture of these compounds would be immunomodulatory to bluegill lymphocytes. The results of this research demonstrate that a diet of these compounds at a total PAH dose of $3.1 \pm 0.01\text{mg}$ for the individual compounds and $1.88 \pm 0.13\text{mg}$ 2-AA, $2.65 \pm 0.15\text{mg}$ 9,10-DMA, and $3.11 \pm 0.28\text{mg}$ 2-MN for the mixture of compounds alters immune response as compared to controls, and are therefore immunomodulatory. All compounds tested were immunomodulatory. The 2-AA diet stimulated enhanced cell proliferation in the absence of any mitogen. The 2-MN diet suppressed lymphocyte response to Con A, but enhanced proliferation in response to calcium ionophore and phorbol ester. The mix diet and the 9,10-DMA diet both suppressed lymphocyte proliferation in response to all mitogen stimulation. In addition to affecting cell proliferation, all PAH diets reduced baseline intracellular calcium levels. The ability of a PAH to affect calcium levels has been related to strength of immunotoxicity. In response to the first hypothesis: the results of this research demonstrate that 2-AA, 2-MN, 9,10-DMA, and MIX are all immunomodulatory to bluegill lymphocytes.

We also proposed the hypothesis that the immunomodulatory effects of a PAH diet are due to the ability to alter lymphocyte signal transduction, and that through the use of mitogens that stimulate different parts of signaling pathways, the affected pathways may become apparent. The results of this research did give information suggesting which signaling pathways are affected by the different PAH. 2-AA may
affect signaling pathways capable of inducing cell stimulation. For example, 2-AA may affect the IL-2R, protein tyrosine kinases, or a combination of proteins in the ras/PKC and calcium pathways. Additionally, 2-AA or a metabolite may act directly on the IL-2 gene in the fashion of a transcription factor to stimulate proliferation. In the case of a diet of 2-MN, the effects may be inhibitory on parts of signaling and stimulatory on other parts. 2-MN may stimulate a small amount of PKC/ras/calcium activation which is effectively masked by inhibition in other parts of the signaling pathway. 2-MN may have inhibitory effects on early signaling proteins such as PTKs or later proteins such as MAP kinases and transcription factors. 9,10-DMA and the MIX diet suppress cell proliferation, but the pathways affected are not apparent. The MIX diet includes 2-MN and 2-AA, which have stimulatory properties, but these effects are masked in the MIX diet. Possibly the interaction of compounds abolishes the 2-AA effect in some way. The results of this research gave some information about the signaling pathways affected by the different PAH, but further research is necessary to identify the specific points in the signaling pathways affected by these compounds.

The hypothesis that a PAH diet would reduce lymphocyte proliferation and increase baseline intracellular calcium was partially correct. 9,10-DMA and the mix diet reduced lymphocyte proliferation in comparison to controls. 2-MN reduced lymphocyte proliferation in response to Con A, but enhanced proliferation in response to phorbol ester and calcium ionophore. 2-AA enhanced lymphocyte proliferative response to all mitogens tested, as well as in the absence of any mitogen. 2-AA was demonstrated to be mitogenic to bluegill lymphocytes. All PAH diets tested resulted
in reduced baseline or resting intracellular calcium. The greatest reduction of intracellular calcium concentration was demonstrated in response to the diets of 2-AA and 2-MN, although the MIX diet and 9,10-DMA also reduced calcium levels slightly. Therefore, parts of the hypothesis in this case were proved correct. Lymphocyte proliferation was reduced, however, not in all instances. Intracellular calcium was not increased as stated in the hypothesis, but rather, intracellular calcium was reduced by the PAH diets.

This study has demonstrated that the PAH 2-AA, 2-MN, 9,10-DMA and a mixture of these compounds are all immunomodulatory to bluegill lymphocytes. These compounds affect immune function in a manner consistent with alteration of signal transduction pathways. A 14-day diet of these compounds alters lymphocyte proliferation and reduces baseline intracellular calcium levels. Further investigation into PAH alteration of signal transduction will continue to be an interesting field of study.
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Appendix I
Effects of PAH on Protein Activation

Introduction

MAP kinases are signaling proteins in lymphocytes that are part of the ras signaling cascade of proteins. Upon TCR stimulation, active ras accumulates, and is responsible for binding and activating Raf-1. Raf-1 subsequently activates MEK, which then activates the "MAP kinases" ERK-1 and -2. ERK-1 and -2 have many cellular substrates, including components of the transcription factor, AP-1. AP-1 binds the IL-2 gene coordinately with NFAT to participate in IL-2 gene transcription. Because MAP kinase is stimulated just prior to transcription factor binding, it makes a good late stage signaling protein to investigate.

Con A was used to stimulate protein activation for 10 minutes prior to cell lysis and extraction of proteins. After stimulation, the proteins were extracted and separated by SDS PAGE electrophoresis, and transferred to a nitrocellulose membrane. The membrane was probed for bound proteins with an antibody to active (phosphorylated) MAP kinase. Anti-active MAP kinase antibody bound proteins in the 60-66 kDa range. The most intense bands on the Western blot (greatest concentration of activated protein) were in the lanes with the control cells and in the lanes with the cells from the 2-AA treated fish. This demonstrates that the cells from the fish fed 2-AA and the cells from the control fish responded with a greater amount of protein phosphorylation (activation), than did the cells from the other PAH diets. The activation of the putative MAP kinase in this assay was anticipated, due to the results observed in the proliferation assay. In the cell proliferation assay, the
proliferation due to Con A was approximately equal in the cells from the 2-AA-fed fish and the cells from the control fish.

**Materials And Methods**

**Preparation of whole cell extracts**

Bluegill lymphocytes were treated with 25μg/ml Con A 10 minutes prior to harvesting. Proteins were harvested on ice in a buffer containing 1.0% Triton, 150mM NaCl, 10mM Tris (pH 7.4), 2mM EDTA, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride, 1μM pepstatin, 50 trypsin inhibitory milliunits of apro tinin, 1% NP-40, 1mM Na₃VO₄ and 1mM NaF and 10μM leupeptin. All buffer components were purchased from Sigma (St. Louis, MO). Protein concentration was determined using a BCA kit (Pierce, Rockford, IL) according to manufacturer's instructions.

**Gel electrophoresis and immunoblotting**

Proteins were loaded 150μg/lane in 12% gels containing SDS. Protein separation was accomplished by electrophoresis under reducing conditions in 15 hours at 50V in a vertical slab gel unit (Hoefer Scientific, San Francisco, CA). Proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA) in 25mM Tris, 192 mM glycine, and 20% methanol. Transfer was done at 35V for 6 hours in a Bio Rad Trans-Blot cell. After the transfer, the nitrocellulose membrane was blocked in 4% milk overnight at 4% C. The blots were probed for bound protein with sheep polyclonal IgG MAP kinase antibodies (specific for ERK-1 and -2). Antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Results were visualized using horseradish peroxidase-conjugated rabbit anti-sheep secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).
Results

In this assay, cells from all of the PAH diet-fed fish and control fish were stimulated with Con A 10 minutes prior to extraction of proteins. Con A was used in an effort to stimulate all signaling proteins and pathways that would be stimulated by TCR with antigen. In this study, we were interested in assessing the activation state of MAP kinase, due to its late stage in the signal transduction pathway (just prior to binding of transcription factors). Activated MAP kinase is phosphorylated on both tyrosine and threonine, and the antibody used in this assay is specific to epitopes present on the activated form of MAP kinase.

The Western blot revealed the presence of two antibody labeled proteins in the 60-66kDa range (Fig. 28). One row of proteins is fairly distinct, the other less so. The distinct bands were estimated at 66kDa, and the more diffuse bands were estimated at 60kDa. MAP kinases, ERK-1 and ERK-2, should be 42-44 kDa. The proteins on the bluegill Western blot were between 60-66kDa. Because the MAP kinase antibody has not previously been tested with fish, and because the MAP kinase protein has not been sequenced in fish, the proteins represented on the Western blot cannot definitively be identified as MAP kinase. Additionally, the bluegill proteins do not appear at the same molecular weight on the immunoblot as would those from rat or mouse. A possible reason for this may be that the gel electrophoresis ran too long at too high of a voltage (15 hours at 50V). The assay was repeated using an active MAP kinase antibody purchased from Promega. In this case, no proteins appeared in the correct range, however, a positive mouse adipocyte control was very apparent, indicating that the antibody was specific for adipocyte MAP kinases. It may not have been cross-
Fig. 28 Phosphorylated (active) MAP kinase in Bluegill peripheral blood lymphocytes, stimulated with 5µg CON A/500,000 cells 10 minutes prior to whole cell lysis. Western blots with anti phospho-MAP kinase (Erk 1/2) antibody revealed the presence of protein bands in the range of 60–66 kDa. 150µg of protein were loaded into each lane. Band intensity is greatest in lanes 1-2 (control) and 3-4 (2-AA), which correlates well with greatest proliferative response to CON A (see previous figure). Lanes 1-2 (control), 3-5 (2-AA), 6-8 (9,10-DMA), 9 (2-MN), 10-11 (MIX).
reactive for bluegill MAP kinases, however. Additionally, there may have been some protein degradation or loss of epitope structure (deactivation) due to thawing and refreezing of the proteins, in order to rerun the assay. In light of these confounding factors, it is possible that the proteins bound by the first anti-MAP kinase antibody (Upstate Biotechnology) in this study are MAP kinase proteins. However, because the Upstate antibody used is to a synthetic tyrosine phosphorylated synthetic peptide, it might be more appropriate to assume that the antibody is bound to a tyrosine phosphorylated protein, in a similar molecular weight range to MAP kinase.

The interesting result from this study is that the darkest protein bands of the putative MAP kinase protein are in the lanes corresponding to the cells from the control fish and to the cells from the fish fed 2-AA. This response is consistent with the results obtained from the cell proliferation assay. In both the proliferation assay and the MAP kinase assay, the 2-AA cells and the control cells show the greatest stimulation. The bands associated with the other PAH diets are not as intense. This is also in agreement with the results from the Con A proliferation assay, in that the cells from the PAH diets, other than 2-AA, did not stimulate proliferation significantly.

The greatest response to Con A stimulation in this study, as evidenced by the immunoblot results, indicate that the 2-AA diet is responsible for stimulating protein activation in excess of the other PAH diets. Although 2-AA is present in the MIX diet, its stimulatory effects are abolished upon combination with the other PAH. Either the interaction between compounds or the synergistic actions of the compounds on signaling proteins, seems to be sufficient to eliminate the stimulatory effects of 2-AA on protein activation.
Discussion

In the lymphocyte, the ras/MAP kinase signal transduction pathway can be stimulated by TCR triggering or IL-2R activation. Activation of ras, by the TCR, stimulates an entire cascade of signaling proteins, which include ras, raf-1, MEK, ERK-1/ERK-2 (MAP kinases), and transcription factors. The MAP kinases, which are present in many cell types, seem to be a convergent point for messages generated by disparate agents. In many cells and tissue types, this pathway can be stimulated by PMA, TCR triggering, IL-2, and other growth signaling proteins (for review see Robbins, 1994). The result of MAP kinase activation in lymphocytes is stimulation of IL-2-binding transcription factors, and cell growth and differentiation. Because MAP kinase activation is a proximal event to transcription factor activation, it is an important step in the ras signaling cascade. As such, its activation is measured as an indicator of late stage signaling cellular response.

Several xenobiotic compounds have been demonstrated to chemically induce MAP kinase activation: chromium in H4-II-E rat hepatocytes (Kim, 1996) hemin and H$_2$O$_2$ in the human T cell line Jurkat (Lander, 1995; Whisler, 1995), and diamide in human neutrophils (Fialkow, 1994). These compounds have in common that they are all oxidizing agents.

Free radicals and redox stress may play a role in mediating signal transduction (Staal, 1994). Cells have protective mechanisms to guard against excessive oxidative stress due to free radical generation. In human peripheral blood mononuclear cells, glutathione acts to buffer the effects of reactive oxidants on sulphydryl oxidation (Reed, 1990). When this protective system is overwhelmed, glutathione is reduced.
and excess thiol oxidation occurs (Nicotera, 1992). Recently, free radicals have been identified as a type of second messenger (Lander, 1997), capable of stimulating signaling by affecting the sulfhydryl sensitive proteins which control signaling pathways. Reactive oxygen (Schreck, 1991) and reactive nitrogen (Lander, 1993b) have been demonstrated to mediate signaling of the transcription factor, NFκB. The iron containing compound, hemin, has been demonstrated to have mitogenic effects on lymphocytes, with free radical generation as a suggested mechanism of activation (Lander, 1993a). HgCl₂ is also mitogenic in lymphocytes, possibly through protein thiol binding. Nitric oxide can directly activate ras \textit{in vitro} by interacting with an important Cys residue in the ras protein (Lander, 1995). Also, hemin, H2O2, and HgCl₂ are able to directly activate ras \textit{in vitro} in Jurkat T cells (Lander, 1995). The ras pathway is also indicated as a target of thiol-oxidizing free radical signaling by Kim (1996), who demonstrates that chromium activates MAP kinase in a PKC-independent fashion in rat hepatocyte cells.

Free radicals have been shown to play important roles in carcinogenesis by directly damaging DNA (Ames, 1993). It is possible that in addition to causing chemical lesions in DNA as a mechanism in carcinogenesis, free radicals may also exert damaging effects on cellular signaling proteins. Reactive nitrenium ions have been shown not only to bind DNA, but also proteins. Clozapine can be oxidized to a reactive nitrenium ion that covalently binds neutrophils and glutathione (Liu, 1995). The aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), found in fried meats, can be N-oxidized to a reactive species that covalently binds glutathione or thiols (Alexander, 1997). The aromatic amine used in this study,
2-aminoanthracene, may undergo activation to a nitrogen centered radical capable of binding DNA and also proteins. If this is the case, the reactive nitrogen radical, the nitrenium ion, may be able to induce effects in the cell in a similar fashion to other oxidizing agents. The reactive metabolite of 2-AA may stimulate activation of the putative MAP kinase by binding and activating MAP kinase itself or one of the signaling proteins upstream of MAP kinase activation.

If it is assumed that 2-AA and the other PAH used in this study act directly on signaling proteins, via their reactive metabolic products, then perhaps these actions may be inhibitory rather than stimulatory in some cases. The oxidizing agent, N-ethylmaleimide, oxidizes specific thiols in human lymphocytes resulting in down regulated PLCγ1 and and PTK activity (Kanner, 1992). DMBA has been demonstrated to impair HPB-All human T cell function by activating tyrosine kinases Fyn and Lck, possibly causing a detrimental increase in intracellular calcium (Archuleta, 1993). A stimulatory action on signaling proteins that results in suppression of function would explain the reduced proliferation in cells from the fish fed 2-MN, 9,10-DMA, and the MIX diet.

**Conclusions**

An anti-active MAP kinase antibody bound to the proteins extracted from Con A-stimulated bluegill lymphocytes. The proteins are likely to be tyrosine phosphorylated, and may represent teleost MAP kinase, although the molecular weight of the proteins differs from other vertebrate MAP kinases. The cells that demonstrated the greatest amount of protein phosphorylation (activation) were isolated from the fish fed 2-AA and from the fish fed the control diet. The 2-AA response may be due to the
interaction of some type of reactive intermediate signal transduction due to metabolites of 2-AA. Additionally, the very faint protein bands observed with the other PAH diets may be the result of depressed proliferation due to reactive intermediates inhibiting signal transduction rather than enhancing it.
Appendix II
SAS Program For Data Analysis

options ls=78 ps=200 nodate nonumber;

data one;

**********************;
* input data set;
**********************;

infile 'a:\helen1.dat';
input y fish diet$ trt$;

**********************;
* get means ;
**********************;

proc sort;
by fish diet trt;
run;

proc means;
var y;
by fish diet trt;
output out=diag1 mean=ymean;
run;

**********************;
* run model ;
**********************;

data model;
set diag1;
lymean=log(ymean);

proc mixed;
class fish diet trt;
model lymean=diet trt diet*trt/p;
lsmeans diet*trt/ pdiff;
make 'predicted' out=diag1 noprint;
make 'lsmeans' out=lsmean noprint;
make 'difs' out=diffl noprint;
run;
* print difflsmeans;

***************;

data diffl;
set diffl1;
format _diff_   6.2;
format _se_   6.2;
proc print;
run;

***************;

* lsmeans;

***************;

data lsml;
set lsmean;
logymean=_lsmean_; 
proc print;
run;

proc gplot;
plot logymean*trt=diet;
symbol1 v=dot;
run;

***************;

* test residuals;

***************;

proc univariate normal data=diagl;
var _resid_; 
run;

***************;

* plot interaction;

***************;

data intplot;
input diet$ trt$ obs;
cards;
Vita

Helen Runnels Connelly was born on January 11, 1964, in Baton Rouge, Louisiana. She attended Runnels School from 1969-1975, and graduated from Episcopal High School in 1981. She entered college at Louisiana State University, where she received a bachelor of science degree in geology in 1985.

Helen worked for Maison Blanche department stores as a buyer until 1992. For a short time after, she worked as an environmental quality specialist for the Louisiana Department of Environmental Quality.

In January of 1993, Helen began working on prerequisite courses to qualify for admission into graduate school. In August, 1993, she and her husband opened Connelly Press and Copy, Inc. where she managed the accounting. In January, 1995, she entered the graduate program at Louisiana State University under Dr. Jay Means, in the Department of Veterinary Physiology, Pharmacology, and Toxicology. Her area of research has been the study of the effects of polycyclic aromatic hydrocarbons on the immune system. While in school, Helen received the Kappa Kappa Gamma Rose McGill Foundation Award and a Career Development Grant from the American Association of University Women. She is one of two nominees from the United States for an award from the International Federation of University Women. Helen is currently a candidate for the Doctor of Philosophy degree, and hopes to use her academic training in the field of environmental consulting.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Helen R. Connelly

Major Field: Veterinary Medical Sciences

Title of Dissertation: Effects of Selected Polycyclic Aromatic Hydrocarbons on Cell Proliferation, Protein Phosphorylation, and Intracellular Calcium Concentration in *Lepomis macrochirus*

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

12/18/97