Apoptosis as a Mechanism of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity

by

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Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirement for the degree of Doctorate of Philosophy in Veterinary Medical Sciences

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November, 1998

Blacksburg, Virginia

Keywords: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), Immunotoxicity, Apoptosis

APOPTOSIS AS A MECHANISM OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD)-INDUCED IMMUNOTOXICITY

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(ABSTRACT)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic environmental pollutant and is well known for its immunotoxic effects, particularly on the thymus. The exact mechanism by which TCDD induces thymic atrophy is not known. In the current study, we investigated whether TCDD triggers apoptosis in the thymocytes and whether Fas and Fas ligand play a role in TCDD-mediated immunotoxicity. Administration of a single dose of TCDD at 0.1, 1, 5 or 50 μg/kg body weight intraperitoneally into C57BL/6 +/+ mice caused a significant dose-dependent decrease in the thymic cellularity; whereas, in the C57BL/6 *lpr/lpr* (*lpr*) (Fas-deficient) and C57BL/6 *gld/gld* (*gld*) (Fas ligand-defective) mice, TCDD failed to induce a decrease in thymic cellularity at doses of 0.1-5 μg/kg body weight. In the *lpr* and *gld* mice, thymic atrophy was seen only at 50 μg/kg body weight of TCDD. Significant apoptosis was detected within 8-12 hours after injection in the wild type mice, whereas, in the *lpr* and *gld* mice apoptosis could not be detected. Upon culturing the thymocytes from TCDD-treated mice for 24 hours *in vitro*, the wild-type cells showed increased apoptosis when compared to the control; whereas, similar cells from *lpr* and *gld* mice did not show apoptosis. Furthermore, TCDD-treatment caused significant alterations in the expression of surface molecules on the thymocytes in the wild-type mice and minimal changes in the *lpr* or *gld* mice. Sera from TCDD-treated wild-type mice also exhibited increased levels of soluble Fas ligand. Also, TCDD-induced apoptosis was inhibited both *in vitro* and *in vivo* by caspase

inhibitors and other inhibitors of apoptosis. Together, the current study demonstrates that TCDD-induced apoptosis plays an important role in thymic atrophy caused by TCDD *in vivo*. Furthermore, phenotypic changes in the density of thymocyte surface molecules may serve as a useful biomarker for chemical toxicity involving apoptosis. The current study also demonstrates that Fas-Fas ligand interactions play an important role in the induction of apoptosis and immunotoxicity by TCDD.

Dedication:

In the memory of my Dad, Balakrishna, who instilled in me the love for learning. This one is for you Dad!
And my Mom, Anuradha, you are a pillar of strength to me.

I would never have made it without you both.

Acknowledgements:

There is a long list of people that I would like to thank. First of all, I would like to thank and express my deepest appreciation to my advisors, Dr. Mitzi Nagarkatti and Dr. Prakash S. Nagarkatti for believing in me and with their support, patience and guidance making me think like a "scientist". Also, I would like to thank my committee members, Dr. S. D. Holladay, Dr. D. J. Blodgett, and Dr. J. C. Lee for their constructive criticism in guiding my research project. Special thanks to Dr. Sam Kacew for serving as my external examiner.

I would also like to thank my parents, my big brother, Ashesh, his wife, Reshma, my aunt, Rohini and little brother, Namdev for their support, love and understanding in helping to make me the first DOC in the whole family!

Special thanks to my dearest friends, Vivek Anand, Dawei Chen, Dr. Mona Hassuneh, Lisa Hudson, Dr. Asimah Rafi-Janejreh, Dr. Isam Janajreh, Vijaylaxmi Mahapatra, and Ahmet Zeytun for their friendship and always helping me keep a sane mind when things went wrong! Thanks to all other members of the Nagarkatti Lab, both past and present, Dr. Denise Hammond-Mckibben, Mark Rhile, Henry Kao, Huimin Xu, Marcus Fiadiero, Mike Bradley, Sara Pryputniewicz, Dr. Lioudmila Sharova, David Scanlan, and Iris Camacho, who made life in the lab enjoyable.

Thanks to my friends and family back in India who kept believing in me.

I would also like to thank the media center and staff in the Vet School for their support and for the friends that I have made in the past four years.

Who could forget the flow cytometry laboratory at the vet school? I practically lived there and without Joan Kalnitsky's help most of this work would have been difficult to accomplish. Special thanks to her for her support and friendship.

Also, thanks to Lucy Gray who always had a kind word for me when I was feeling down and kept cheering me on.

Special thanks to the animal care facility at the vet school and in Biology for taking care of the numerous mice and keeping them happy! Thanks also to, Chris Wakley, Mary Nickle, Susan Anderson, Judy McCord, Amy Wood and Jenny Sutphin.

I would also like to thank Dr. Lee's office for providing financial support and to Linda Price for all her help.

I would also like to thank American Cancer Society, National Institutes of Health, NSF/EPA, Virginia Academy of Sciences, Sigma-Xi Grants in Aid of Research, and the Graduate Research Development Program for funding this research.

Last but not the least, I would like to thank God for giving me the strength and encouragement to believe in myself and follow my dreams.

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List of Abbreviations

Ah, aryl hydrocarbon

APC, antigen presenting cell

CD, cluster of differentiation

CMI, cell mediated immunity

CTL, cytotoxic T lymphocyte

DN, double negative

DNA, deoxyribonucleic acid

DP, double positive

DRE, dioxin responsive element

FcR, Fc receptor

FITC, fluorescein isothiocyanate

gld, generalized lymphoproliferative disorder

i.p., intraperitoneally

IFN, interferon

Ig, immunoglobulin

IL, interleukin

JAM test, a test for DNA fragmentation

LN, lymph node

lpr, lymphoproliferative disorder

mAb, monoclonal antibody

MHC, major histocompatibility complex

MFI, mean fluorescent intensity

NK cell, natural killer cell

PBS, phosphate buffered saline

PE, phycoerythrin

RPMI, Roswell Park Memorial Institute culture medium

SD, standard deviation

SEM, standard error of the mean

SRBC, sheep red blood cell

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

TCR, T cell receptor

TdT, terminal deoxynucleotidyl transferase

TGF, transforming growth factor

Th, T helper cell

TNF, tumor necrosis factor

TUNEL, TdT-mediated nick end labeling

Chapter 1: General Introduction and Review of Literature

The Immune System:

The origin of immunology is attributed to Edward Jenner who discovered in 1796 that cowpox, or vaccinia, induced protection against human smallpox. The discoveries by Koch and others in the 19^{th} century stimulated the extension of Jenner's strategy of vaccination to other diseases. Louis Pasteur devised a vaccine against cholera in chickens and developed a rabies vaccine. In 1890, Emil von Behring and Kitasato discovered that the serum of vaccinated individuals contained substances which they called antibodies that specifically bound to the relevant pathogen. Immunity means protection from disease and cells responsible for immunity constitute the immune system and the coordinated response to foreign substances is called the immune response. Healthy individuals protect themselves against microbes through physical barriers, phagocytic cells and eosinophils in the blood and natural killer cells. All of these defense mechanisms are present prior to exposure to foreign substances. These constitute the components of the natural or innate immunity. A specific immune response, such as the production of antibodies to a particular pathogen, is known as an adaptive or acquired immune response. The immune system is an extremely complex but remarkably adaptive defense system, which has evolved to protect invertebrates from invading pathogens. Functionally, an immune response can be divided into two interrelated activities - recognition and response. It is a complex collection of organs, tissues and cells that are capable of a highly specific and adaptive response (Hildemann, 1984). The central cell of the immune system is the lymphocyte. There are approximately 10^{12} lymphocytes in humans and these lymphocytes continuously recirculate between the blood and lymph and the various lymphoid organs, thereby providing a high degree of cellular integration to the immune system as a whole.

Organs of the Immune System: A number of morphologically and functionally diverse organs have various functions in the development of an immune response. These organs can be divided on the basis of function into primary and secondary lymphoid organs. The primary organs provide appropriate microenvironments for lymphocyte maturation and the secondary organs trap antigen from defined tissues and vascular spaces and provide sites where mature lymphocytes can interact effectively with that antigen. Immature lymphocytes generated during hematopoiesis mature and become committed to a particular antigenic specificity within the primary lymphoid organs. Only after a lymphocyte has matured within a primary lymphoid organ is the cell immunocompetent. In the mammals, the primary lymphoid organs are the bone marrow, where the B cell maturation occurs and the thymus, where the T cell maturation occurs. A variety of peripheral lymphoid organs exist each uniquely suited to trap antigen from defined tissues or vascular spaces and to provide sites where mature, immunocompetent lymphocytes can interact effectively with antigen from the intracellular tissue fluids, whereas the spleen filters blood-borne antigens. The respiratory and gastrointestinal tracts possess aggregrations including Peyer's patches, tonsils, adenoids, and the appendix which trap antigens entering through various mucous membrane surfaces (Kuby, 1994).

The thymus is a central lymphoid organ that allows for T cell maturation. It is present in the upper anterior thorax, just above the heart. It is bi-lobed and each lobe is differentiated into an outer cortical region, the thymic cortex and the inner medulla. The thymic stroma arises early in embryonic development from the endodermal and ectodermal layers of the embryonic structures known as the third pharangeal pouch and the third branchial cleft. The bone marrow stem cells are differentially distributed between the thymic cortex and medulla. The cortex contains only immature thymocytes and scattered macrophages, whereas more mature thymocytes, along with dendritic cells

and most of the macrophages, are found in the medulla. The rate of T cell production by the thymus is greatest before puberty. After puberty, the thymus begins to shrink and production of new T cells in adults is lower although it remains functional throughout life.

Changes occur in various membrane glycoproteins and receptors during thymocyte maturation. Progenitor T cells begin to express a membrane marker called Thy-1, which is a marker for all thymus-derived lymphocytes in the mouse. These markers are called the clusters of differentiation (CD) and are used to characterize cells. The earliest fetal thymocytes lack detectable CD4 and CD8 and are known as double-negative cells. These cells then differentiate into cells expressing both CD4 and CD8 along with CD3 and T-cell receptor (TCR). This is followed by the loss of CD4 or CD8 and results in the commitment of the cell into a helper T cell ($CD4^+CD8^-$) or a cytotoxic T cell ($CD4^-CD8^+$). The CD3 molecule is retained throughout the lifetime of the T cell as is the TCR (Golub and Green, 1991).

The selection of T cells in the thymus involves two processes: (1) positive selection of thymoyctes bearing receptors capable of binding self-MHC molecules which results in MHC restriction and, (2) negative selection by elimination of thymocytes bearing high-affinity receptors for self-MHC molecules alone or self-antigen presented by self-MHC which results in tolerance. Negative selection leads to the removal of the cells by apoptosis or programmed cell death. It is in this manner that T cells are “educated” to discriminate between self and non-self. Thus, of the cells entering the thymus, only a small proportion leave the thymus as mature T cells.

The spleen is a large, ovoid secondary organ situated high in the left abdominal cavity. It is adapted to filtering blood and trapping blood-borne antigens, and thus can respond to systemic infections. The spleen has a compartmentalized structure. The compartments are of two types, the red pulp and the white pulp which are separated by a marginal zone.

The red pulp consists of a network of sinusoids populated with macrophages and numerous erythrocytes and is the site where the old and defective red blood cells are removed. The white pulp surrounds the arteries, forming a periarteriolar lymphoid sheath populated mainly by T lymphocytes. The marginal zone is rich in B cells organized into primary lymphoid follicles. The spleen also serves to allow the interaction of T and B cells with macrophages and antigen to elicit immune responses.

The lymph nodes are encapsulated bean-shaped structures containing a reticular network packed with lymphocytes, macrophages and dendritic cells. They are clustered at junctions of the lymphatic vessels and are the first organized lymphoid structures to encounter antigens that enter the tissue spaces. Morphologically, a lymph node can be divided into three concentric regions: the cortex, which is the outer most layer and contains mostly B cells and macrophages arranged in the primary follicles; the paracortex lies just below the cortex and is populated with T lymphocytes and dendritic cells that have migrated from the tissues to the node and the last region is the medulla, which is more sparsely populated with lymphocytes but many of these are plasma cells actively secreting antibody molecules. Any particulate antigen gets trapped by the cellular network of phagocytic cells and dendritic cells and provides an ideal microenvironment for lymphocytes to encounter and respond to trapped antigens.

In humans, hematopoiesis, the formation and development of red and white blood cells from stems cells begins in the yolk sac in the first weeks of embryonic development. As gestation continues, the bone marrow becomes the major hematopoietic organ. Bone marrow is present in almost all bones of the body but is most abundant in the long bones. Early in hematopoiesis, a pluripotent stem cell differentiates along one of the two pathways, giving rise to either a lymphoid stem cell or a myeloid stem cell. Subsequent differentiation of lymphoid and myeloid stem cells generates committed progenitor cells for each type of mature blood cell. The lymphoid stem cell generates T and B progenitor

lymphocytes and the myeloid stem cell generates progenitor cells for erythrocytes, neutrophils, eosinophils, basophils and platelets. When the appropropriate growth factors are present, these progenitor cells proliferate and differentiate into the corresponding mature red and white blood cells (Janeway, 1997).

Cells of the Immune System: The lymphocytes are the white blood cells responsible for the immune response. They can be broadly divided into three populations: T cells, B cells and the natural killer cells. These populations can be distinguished by function and their expression of cell surface markers. The T cells derive their name from their site of maturation in the thymus. The T cell receptor for antigen is structurally distinct from the immunoglobulin but does have some structural features in common with the immunoglobulin molecule, most notably in the structure of its antigen-binding site. The T cell recognizes the antigen only when its associated with a self-molecule encoded by genes within the major histocompatibility complex (MHC). The T cells are characterized by their expression of TCR/CD3 complex. The TCR consists of a heterodimer of polypeptides held together by disulfide bonds. The chains of the TCR consist of a number of regions including a highly polymorphic region known as the variable (V) region that enabled T cells to interact specifically with many distinct antigens which may be encountered in the body. T cells may express a TCR consisting of α and β chains or a TCR composed of γ and δ chains. The group of T cells bearing the $\gamma\delta$ TCR are $CD4^-$ $CD8^-$ cytotoxic T cells but their exact role in the immune system is not known. The T cells that express the CD4 membrane molecule recognize antigen associated with MHC Class II molecules, whereas, T cells that express CD8 on their membrane surface recognize antigen associated with MHC Class I. Thus, the expression of CD4 versus CD8 corresponds to the MHC restriction of the T cell. The $CD4^+$ T cells function as T helper (T_h) cells and are class II retricted. The $CD8^+$ T cells function as T cytotoxic (T_c) cells and are class I restricted. Class I MHC is expressed on all nucleated cells and thus

allows T_c to destroy any virally infected cell in the body. Class II MHC is present on the cell surface of antigen presenting cells (APCs).

There are two main routes of antigen presentation that occur. Antigen presentation with class II MHC involves the uptake of antigen by endocytosis or by binding to surface immunoglobulins in the case of B cells. The antigen is then fragmented by proteolytic enzymes and becomes associated with class II molecules. This complex is then presented on the surface of the cell for presentation to $CD4^+$ T_h cells. In case of class I MHC association, the antigen is primarily endogenous, such as a viral protein. These antigens are degraded in the cytoplasm and then associated with class I MHC molecules for recognition by $CD8^+$ T_c cells. Macrophages assume the major role as APCs but other cells such as B cells and dendritic cells can also function as APCs. This is the main difference between humoral and cell-mediated immunity.

The B cells derive their name from their site of maturation in the bursa of Fabricus in birds and the bone marrow in mammals. The B cells are categorized by the presence of immunoglobulins (Ig) or antibodies (Ab) on their membrane surface. These antibodies on the cell surface are endogenously synthesized and act as antigen specific receptors. There are five classes of immunoglobulins including IgM, IgD, IgG, IgE and IgA. Most cells express both IgM and IgD on their cell surface. B cells also express complement receptors and Fc receptors to which antibody may bind. The B cell is capable of binding to soluble antigen, whereas the T cell is restricted to binding antigen displayed on the self-cells.

Another major group of immune cells is the natural killer (NK) cells. These are $CD3^-$, $CD16^+$, $CD56^+$, $CD2^+$ large granular lymphocytes involved in the non-specific killing of tumor cells as well as lysis of virally infected cells. The NK cells bear the NK 1.1 surface molecule. Unlike T_c, NK cells are MHC-unrestricted and can effectively lyse

target cells lacking class I MHC. The NK cells therefore play an important role in immunosurveillance by destroying tumor cells that lack class I MHC expression and cannot be effectively lysed by cytotoxic $CD8^+$ T cells.

The granulocytes are classified as neutrophils, eosinophils or basophils based on their cellular morphology and cytoplasmic staining characteristics. Neutrophils are the first to arrive at a site of inflammation and are active phagocytic cells that contain lytic enzymes and bactericidal substances within the primary and secondary granules. Eosinophils, like neutrophils, are phagocytic cells that migrate from the the blood into the tissue spaces. Their phagocytic role is significantly less important than that of neutrophils and their major role is in defense against parasitic organisms by release of substances to counter the effects of histamines produced and thus reduce inflammation. Basophils are not phagocytic and function by releasing pharmacologically active substances contained within their cytoplasmic granules such as serotonin, heparin and histamine (Kuby, 1994). They have a major role in allergic responses during which they release the contents of their granules.

Mast cells can be found in a variety of tissues including skin, connective tissues of various organs and mucosal epithelial tissue of the respiratory, genitourinary and digestive tracts. Like basophils they have large numbers of cytoplasmic granules containing pharmacologically active substances which are released during allergic reactions.

Dendritic cells are cells that are covered with long membrane like processes resembling dendrites of nerve cells. They are found in lymphoid and non lymphoid tissues of the body. They express high levels of class II MHC molecules and function as important antigen-presenting cells for T cell activation.

The monocytes consist of circulating monocytes in the blood and macrophages in the tissues. They perform two main functions: the first as phagocytic cells and the second as antigen presenting cells (APCs). Differentiation of a monocyte into a tissue macrophage involves a number of changes. Macrophages are dispersed throughout the body and are found in tissues such as the lung, brain, spleen and kidney. Macrophages are normally in a resting state but in the course of an immune response get activated. Phagocytosis of particulate antigen serves as the initial activating stimulus. Activated macrophages have increased phagocytic activity, increased secretion of inflammatory mediators and an increased ability to activate T cells. This increased activity helps to eliminate potential pathogens. Activated macrophages also express higher levels of class II MHC molecules allowing them to function more effectively as APCs to $CD4^+$ T cells. The macrophages and T_h cells exhibit an interacting relationship during the immune response with each facilitating activation of the other. Monocytes and macrophages possess lysosomes which contain hydrogen peroxide, oxygen free radicals, peroxidase, lysozyme and various hydrolytic enzymes that digest the ingested material and eliminate them in a process called exocytosis. These cells have strong adherent properties aided by the expression of complement and Fc receptor. Macrophages also produce important cytokines such as interleukin-1 (IL-1), prostaglandins, tumor necrosis factor (TNF), interferon (IFN) and colony stimulating factor (CSF). These monokines are involved in stimulating other cells of the immune system and also play a role in inflammation processes (Roitt, 1996).

Immune responses can be divided into humoral and cell mediated responses depending on the way the antigen is presented to the lymphocyte (Roitt, 1996). The humoral branch of the immune system involves interaction of B cells with antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibodies function as effectors of the humoral response by binding to the antigen and neutralizing it or facilitating its elimination. This is accomplished through the variable domain that,

like the TCR, is highly polymorphic and thus antibody to any antigen that is encountered in the body may be produced. Antibodies express a constant region that is involved in binding to immune cells that express Fc receptor and thereby, aid effector cells in target recognition. Additionally, the constant region is essential in the complement pathway. Antibodies also act by directly neutralizing bacterial toxins and can coat bacteria to enhance phagocytosis by macrophages. This binding also activates the complement system, resulting in the lysis of the foreign organism. Antibodies consist of two identical light chains and two identical heavy chains, each chain having a constant and variable region. There are five major classes of immunoglobulins that are distinguished by their heavy chains, namely, IgG, IgM, IgD, IgE and IgA. The IgG antibody makes up the bulk of immunoglobulin in the human immune system and is the major antibody of secondary immune responses and the only antibody that has anti-toxin capability (Roitt, 1996). IgA is found mostly in secretions such as tears, saliva, colostrum and mucus. IgM is present in highest concentration in the blood and is the first antibody to appear in the immune response. IgE is the antibody responsible for immediate hypersensitivity reactions although it is found in trace amounts. IgD is found bound to the membranes of B cells and its exact function is not clear.

The cellular branch of the immune system involves antigen presentation and activation of T cells. Both T_h cells and cytotoxic T lymphocytes (CTLs) serve as effectors of the cell mediated response. It involves the destruction of intracellular parasites, tumor responses, graft rejection and delayed type hypersensitivity. These types of immune responses include not only T cells but the cooperation of numerous immune cells as well as the production of various lymphokines. Lymphokines can produce more efficient immune responses and also can down regulate the immune system.

T cells recognize antigen with the help of accessory cells such as APCs in association with the MHC molecules on their cell surface. The role of T_h cells is crucial to the

immune response. These cells display an important regulatory role in the immune system. Upon activation, T_h cells secrete a number of chemical substances known as lymphokines that can mediate cellular responses. These molecules include a class of cytokines that are called interleukins (IL). Interleukins such as, IL-2, IL-4, interferon gamma (IFN-γ) and IL-6 are important cytokines produced by T_h cells. The T_h cells are also needed in the production of antibodies by B cells. The development of a cytotoxic response involves both sets of T cells. The T_h cells are needed to produce IL-2 in order to drive differentiation into T_c cells and enhance their cytotoxicity. The T_c cells bind targets bearing both antigen and the class I MHC molecule. This is followed by the release of cytotoxic granules that contain perforins, lymphotoxins and granzymes. These substances disrupt the membrane of the target cell leading to its lysis. These functions are essential in the destruction of virally infected cells and some tumors. The humoral and cell mediated immune reactions do not necessarily occur independently of each other and often an immune response involves both of these mechanisms.

Apoptosis:

Cells can die in either of the two ways. Necrosis, where physical or chemical injury, such as the deprivation of oxygen, or membrane damage with antibody and complement leads to cell disintegration. The dead or necrotic tissue is taken up and degraded by phagocytic cells which eventually clear the damaged tissue and heal the wound. The other form of cell death is known as programmed cell death or apoptosis. It was discovered in 1842 by Carl Vogt. The term apoptosis was coined by Kerr, Wyllie and Currie in 1972 (Kerr *et al*., 1972). It is a normal cellular response that is crucial in the tissue remodeling that occurs during embryogenesis, metamorphosis, normal tissue turnover and endocrine-dependent tissue atrophy in all multicellular animals. In the thymus, most thymocytes die an apoptotic death when they fail positive selection or are

negatively selected as a result of recognition of self antigens. The first changes seen in apoptotic cell death are DNA fragmentation, disruption of the nucleus and alterations in the cell morphology. The cell then destroys itself from within, shrinking by shedding membrane bound vesicles and degrading itself until little is left. The hallmark of this type of cell death is the fragmentation of nuclear DNA into fragments that are multiples of 200 base pairs through the activation of endogenous nucleases that cleave the DNA between the nucleosomes. The apoptotic bodies are then phagocytosed by the macrophages (Janeway, 1997; Wyllie *et al*., 1980).

In 1989, two groups independently isolated mouse-derived antibodies that were cytotoxic for various human cell lines (Trauth *et al*., 1989; Yonehara *et al*., 1989). The cell surface protein recognized by the antibodies were designated as Fas and APO-1, respectively. Cloning of the APO-1 antigen cDNA indicated that APO-1 antigen is identical to Fas. A comparison of the amino acid sequence of Fas with all protein sequences revealed that Fas is a member of the TNF/NGF receptor family (Itoh *et al*., 1991; Nagata , 1997). The extracellular regions of the members in this family are rich in cysteine residues and can be divided into three to six sub-domains. The amino acid sequence in this region is relatively conserved, whereas the cytoplasmic region is not. Tissue distribution of the Fas mRNA has been examined in adult mice and the thymus, heart, liver and ovary have been shown to abundantly express Fas mRNA. In the thymus, most thymocytes express Fas as do activated T and B cells. Signaling by Fas leads to apoptotic cell death. Triggering this cell death requires the cross-linking of Fas either with antibodies to Fas, with cells expressing Fas ligand (FasL) or with purified FasL and therefore Fas is called a receptor for a death factor.

Fas ligand (FasL) was identified as a membrane protein using Fas-Fc fusion protein which inhibited the Fas-dependent cytotoxic T cell (CTL) activity. The purifed FasL has specific cytolytic activity against cells expressing Fas (Suda and Nagata, 1994),

suggesting that a single protein (FasL) is sufficient to induce apoptotic cell death by binding to Fas. The amino acid sequence of FasL revealed that FasL is a member of the TNF family. It is expressed abundantly in the testis, moderately in the small intestines and weakly in the lungs, whereas, little expression of FasL mRNA was observed in the liver, heart and ovary where Fas is abundantly expressed. FasL is also found in the soluble form in the body under abnormal conditions. The extracellular domain of the FasL gets cleaved to release this soluble form which can be found in the serum.

Spontaneous mouse mutants *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) carry autosomal recessive mutations on mouse chromosome 19 and 1, respectively. The *lpr* and *gld* mice develop lymphadenopathy and splenomegaly and produce large amounts of IgG and IgM antibodies including anti-DNA and rheumatoid factor. They develop nephritis or arthritis and die around 5 months of age. Lymphocytes that accumulate in the lymph nodes and spleen of these mice express the T cell marker, Thy-1 and B cell marker, B220. These cells also express a rearranged TCR but not a rearranged IgG gene. The CD4 and CD8 antigens, which are usually expressed in mature T cells, are not expressed in the lymphocytes that accumulate in the *lpr* and *gld* mice (Watanabe-Fukunaga *et al*., 1991).

Although *lpr* and *gld* are non-allelic mutations, they show the similar phenotypes for lymphadenopathy and splenomegaly. The loss of function mututation of Fas and FasL causes activated lymphocytes to accumulate in these mice and this produces autoimmune disease. It has been shown that *lpr* and *gld* are mutations in the genes encoding interacting proteins: *gld* may affect a soluble or membrane cytokine, whereas the *lpr* may affect its receptor. The Fas gene was mapped to a location near the *lpr* locus on mouse chromosome 19 and it was found that the lpr mice expressed little or no Fas mRNA. Characterization of the Fas gene in the *lpr* mice indicated that an early transposable element (ETn), a variety of the mouse retrovirus, is inserted into intron 2 (Adachi *et al*., 1993). This caused the transcription of the Fas gene to be impaired in

these mice. The FasL gene was similarly mapped on mouse chromosome 1 where the *gld* mutation is localized. There is no rearrangement of the FasL gene in the *gld* mice, but there is a point mutation near the carboxy terminus of the coding region. This mutation abolishes the ability of FasL to bind to Fas. Thus the Fas-FasL interactions lead to a cascade of intracellular reactions which eventually lead to the induction of genes for mediating apoptotic cell death. This ligation leads to the recruiting of the cytosolic adapter protein FADD (Fas associated death domain) to the activated receptor. The signaling complex arranged at these death receptors include FLICE (FasL associated interleukin-1 converting enzyme) and caspase-8 which associates with the death effector domain (DED) (Boldin *et al.*, 1995).

Several of the biochemical events that contribute to apoptotic cell death have been recently elucidated. The elucidation of the apoptotic pathway in C. elegans has been helpful to better understand apoptosis signaling pathways in higher eukaryotes. Genetic evidence in nematodes has identified both positive and negative regulators of apoptosis. The key pro-apoptotic gene, ced-3, encodes a cysteine protease that is related to mammalian interleukin-1 converting enzyme (ICE), the first identified member of a new family of cysteine proteases with the distinguishing feature of a near absolute specificity for aspartic acid in the S1 subsite. It has been termed as Caspase-1 (Cysteine aspartase) (Nicholson and Thornberry, 1997; Villa *et al.*, 1997). All the known caspases are synthesized as zymogens that require cleavage adjacent to aspartates to liberate one large and one small subunit, which associate into an $\alpha_2\beta_2$ tetramer to form the active enzyme. This requirement enables the caspases to activate other caspases thereby setting the stage for an amplifying cascade. Several classes of caspases have been indentified and most of them are important for the promotion of the death pathway in mammals. They are divided into two classes based on their N-terminal prodomains. Caspases-1,-2,-4,-5,-8 and -10 have long prodomains, whereas, caspases-3,-6,-7 and -9 have short prodomains. The long prodomain appears to be involved in targeting and regulating activation. The

caspases with a short prodomain act at the downstream end of cascade to cleave substrates. Caspase-8 (FLICE/MACH) is an activator caspase capable of activating all other caspases and therefore can act at the top of the hypothetical cascade. Others involved are caspase-10 (Mch4) and caspase-2 (Ich-1/Nedd2). Effector caspases such as caspase-3 (Yama/CPP32/apopain), caspase-6 (Mch2) and caspase-7 (Lap3/Mch3/CMH1) are responsible for the cleavage of many substrates that result in the morphological and biochemical changes associated with apoptosis (Cohen, 1997; Villa *et al*., 1997; Livingston, 1997). Calpains, on the other hand are calcium-dependent and papain-like proteases (endopeptidases) which are modulatory proteases and not digestive proteases. They play a role in cell shape and adhesion, gene regulation, signal transduction, homeostasis and platelet aggregation and long term potentiation. Disruption of the calpain-mediated ion homeostasis or cytoskeleton leads to cell death via apoptosis (Molinari and Carfoli, 1997). Several classes of reversible and irreversible inhibitors have been described for cysteine proteases. The tetrapeptide, Ac-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-CMK) is an inhibitor of caspase-1; whereas, Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO) is an inhibitor of caspase-3. Inhibitors of calpains are calpain inhibitor 1 and 2 which inhibit by binding to the calcium binding domain of the enzyme. Some naturally occurring inhibitors of caspases are viruses such as the poxvirus and baculovirus which have evolved specialized gene products (e.g. CrmA (cytokine response modifier A) from cowpox virus, p35 from baculovirus). The central role of caspases in the biochemical events that mediate the apoptotic stimuli has been well established. A broad range of potential therapies may result from the design of inhibitors of the caspases in treating human diseases. Another inhibitor of apoptosis aurintricarboxylic acid (ATA), a triphenyl-methyl compound, has been shown to inhibit DNA strand breaks.

Immunotoxicology:

The field of toxicology that deals with the effects of chemicals on the immune system is called immunotoxicology. This area of research strives to understand how the immune system is involved in the metabolism and elimination of xenobiotics, or foreign compounds from the body. It has been implicated that the immune system is a target organ of toxic insult following chronic or subchronic exposure to certain chemicals or therapeutic drugs (xenobiotics). Because the immune system is vital in host defense mechanisms, any compound that is able to alter immune functions may directly or indirectly affect the survival of the host. Therefore, it is important to study any chemical encountered in the environment or taken therapeutically that may modulate the immune system.

Awareness of immunotoxicology was stimulated by a comprehensive review by Vos in 1977, in which he provided evidence that a broad spectrum of xenobiotics alter immune responses in laboratory animals and subsequently may affect the health of exposed individuals. Sharma and Zeeman published their findings about a number of environmental contaminants in 1980 and suggested several possible mechanisms by which immunotoxic agents may act. Many other investigators have also contributed to the evolution of this field including Dean and Luster who have compiled numerous volumes on the classification and effects of various immunotoxic compounds (Burrell *et al*., 1992, Dean *et al*., 1994).

While the effects of some chemicals may be obvious in that they cause severe organ or tissue damage, the effects of xenobiotics on the immune system often occur at doses that do not otherwise show toxic symptoms. Toxicological manifestations in the immune system following xenobiotic exposure in experimental animals appear as alterations in lymphoid tissue, peripheral leukocytes, or bone marrow; impairment of cell functions;

and increased susceptibility to infectious agents and tumors. The undesirable effects may be: (1) those determined by immune suppression; (2) those determined by immune dysregulation (autoimmunity); and (3) those determined by the response of immunologic defense mechanisms to the xenobiotics (hypersensitivity) (Dean *et al*., 1994). Although many of these effects result in the suppression of immune functions, it is possible for xenobiotics to stimulate the immune system in a potentially unfavorable fashion.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD):

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the most toxic member of a class of planar, halogenated chemicals or hydrocarbons (HAH). It has no known industrial or commercial use and has been produced as an unwanted byproduct of certain industrial processes and combustion. It is produced by a number of primary sources including chemical, thermal, photochemical enzymatic reactions. Dioxin has been produced during the production of certain chlorinated phenols and their derivatives as a result of high temperature pyrolysis and combustion of organic compounds containing halogens. Chlorine bleaching of paper pulp has also led to the production of dioxin in paper products such as coffee filters, milk cartons, facial tissue and diapers (Rappe, 1991). TCDD is also produced during the incineration of wood, garbage, plastics and other wastes. Dioxin can also be found in consumer products such as chlorinated herbicides, polychlorinated biphenyls and chlorinated phenol-containing products and contaminated paper goods. Dioxin is extremely stable both to environmental and biological breakdown, leading to its persistence in the environment and its bioaccumulation in the food chain. It is highly lipophilic and water insoluble and concentrates in fatty tissues of birds, reptiles, fish and mammals (Banbury Report 18, 1984 and Banbury Report 35, 1991).

Dioxin causes a broad range of effects, some of which are species specific. Dioxin is described as the most toxic man-made chemical because of the low doses which cause lethality in certain animal species such as the guinea pig. Its toxicity has been widely studied and characterized. It causes severe wasting syndrome with a loss of body mass. Atrophy of the lymphoid tissue such as thymus and spleen and of the testes occurs at acutely toxic doses in adult animals. Reduction in thymic weight appears to be a very sensitive parameter of TCDD exposure. This finding suggested that TCDD may alter immune responses. Hyperplastic and metaplastic changes are also characteristic of certain epithelial tissue. The most commonly described human symptom is chloracne in which hyperplasia and hyperkeratinization of the epidermis has been noted (Poland and Kende, 1976). It has been called the hallmark of dioxin toxicity and occurs following dermal or systemic exposure in sensitive species. Other effects in laboratory animals due to exposure to TCDD include reproductive toxicity, teratogenicity, endocrine effects, dermal lesions and the induction of several hepatic xenobiotic metabolizing enzymes (Poland and Knutson, 1982; Thomas and Faith, 1985; Davis and Safe, 1988). Cleft palate is a major structural abnormality following prenatal exposure to TCDD only in the mouse at doses which are not fetally or maternally toxic. The developing immune system is also affected leading to altered differentiation of lymphocytes. The carcinogenicity of TCDD has also been studied and it has been shown to cause tumors at multiple sites. Dioxin is highly immunotoxic in the mouse. It has been shown to have profound effects on the immune system even at very low levels that do not induce organ toxicity. Studies have shown that TCDD can suppress cell mediated immunity (CMI) and humoral immunity involving antibody (Ab) production by B lymphocytes (Faith and Moore, 1977; Faith and Luster, 1979; Sharma *et al*., 1978). Other immunomodulatory effects of TCDD include suppression of B cell differentiation (Fine *et al*., 1988), dose-dependent suppression of cytotoxic T lymphocytes (Thigpen *et al*., 1975), thymic atrophy (Thomas and Faith, 1985), impairment of delayed type hypersensitivity responses and increased susceptibility to certain infectious agents (Clark *et al*., 1981).

Though it is clear that dioxin alters these immune functions, the mechanism of its toxicity is unclear.

The biochemical effects of TCDD represent a molecular and cellular response to the chemical. These effects can be grouped into three classes: (a) altered metabolism resulting from changes in the enzyme levels; (b) altered homeostasis resulting from changes in hormones and their receptors; and (c) altered growth and differentiation resulting from changes in growth factors and their receptors. Many of these effects are tissue specific (Birnbaum, 1994).

Biological responses to TCDD as well as other foreign chemicals or xenobiotics are regulated by the major histocompatibility complex (MHC) and the Ah (aryl hydrocarbon) locus (Nerbert and Negishi, 1982). The Ah locus is responsible for the induction of metabolizing enzymes such as cytochrome P_1-450 and aromatic hydrocarbon hydroxylase (AHH). Many of the biochemical and toxic effects of halogenated aromatic hydrocarbons (HAH) appear to be mediated via binding to an intracellular protein known as the aryl hydrocarbon (Ah) or TCDD receptor (Carlstedt-Duke *et al.,* 1979; Gasiewicz *et al*., 1984). This is a high affinity binding protein present in low numbers per cell and has been found in most tissues, although the number varies. The Ah receptor has a basic helix-loop-helix domain that allows interaction with DNA and interacts with DNA in the form of a heterodimer with another basic helix-loop-helix protein. TCDD action can be thought of as involving three separate steps: (a) recognition of the signal; (b) transduction of the signal; and (c) response. The Ah receptor activation follows stereospecific ligand binding to TCDD or a related compound. This interaction is highly specific and the form of the ligand binding subunit of the Ah receptor that binds to TCDD is not an isolated peptide, but a part of a multimeric complex. Two heat shock proteins (HSP90) are involved in this ligand-binding complex (Perdew, 1988). Once TCDD is bound to the receptor, the other proteins dissociate and the Ah receptor-TCDD

complex is translocated into the nucleus. This reaction requires the interaction with a protein called the "arnt" (aryl hydrocarbon receptor nuclear translocating) protein. The arnt protein dimerizes with the ligand binding subunit to form the DNA binding species. The activated receptor-ligand complex binds to specific sites on DNA and appears to function as a transcriptional enhancer. These specific sites are known as the "dioxin responsive enhancer" (DRE) sites and are located in the 5' region upstream of the structural gene for the cytochrome. Binding to this site induces the transcription of the structural genes encoding mRNA for CYP1A1 enzyme activity (cytochrome P_1-450) as well as the expression of additional unidentified genes, the products of which are hypothesized to mediate HAH toxicity (Clark *et al*., 1981; Nebert and Negishi, 1982; Nebert, 1989). Studies focusing on the Ah locus have used aryl hydrocarbon hydroxylase (AHH) to establish the phenotype of the Ah locus in particular strains of inbred mice. The different alleles code for Ah receptors that differ in their ability to bind TCDD and thus help explain the different sensitivities of various inbred mouse strains to TCDD toxicity. Half of all the inbred strains of mice are Ah-responsive meaning that enzymes such as cytochrome P_1-450 are inducible by polycyclic aromatic hydrocarbons. In non-responsive strains of mice cytochrome P_1-450 is non-inducible or less inducible. The Ah^b has been determined to be the dominant allele for responsiveness and Ah^d as the recessive one. Thus, experimental results have shown C57BL/6 (Ah^b/Ah^b) to be a TCDD-responsive strain and DBA/2 (Ah^d/Ah^d) to be unresponsive (Nebert and Negishi, 1982; Nebert, 1989). TCDD could induce enhanced suppression in Ah^{bb} mice when compared to Ah^{dd} mice (Kerkvliet *et al*., 1990). It should be noted that, although induction of AHH and associated enzymes has been shown to be related to the Ah-R (Safe, 1986; Whitlock, 1990; Okey *et al*., 1983). Okey *et al*. (1983) demonstrated that the Ah-R was necessary but not sufficient for the induction of AHH.

Responsiveness to TCDD is linked to segregation with the Ah locus. Clark et al. (1983) showed that suppression of CTL induced by TCDD segregated with the Ah locus

(Whitlock, 1987; Clark *et al*., 1983; Nagarkatti *et al*., 1984). Poland and Grover (1980) found that TCDD-induced thymic atrophy segregated with the Ah-receptor (Ah-R).

Vecchi *et al*. (1983) were the first to report that the antibody response to sheep red blood cells (SRBCs) was differentially suppressed by TCDD in C57BL/B6 mice as compared to DBA/2 mice. *In vitro* studies by Davis and Safe (1988) indicated that TCDD and its congeners produced a concentration-dependent suppression of the anti-SRBC response using cells from either C57BL/6 or DBA/2 mice. In contrast, it has been observed that the toxicity of TCDD may be mediated by Ah-independent mechanisms. Studies using 2,7-DCDD, a TCDD congener with a weak affinity for Ah-R, showed a suppressed Ab response to SRBC with no effect on the thymus or AHH activity while TCDD caused thymic atrophy and increases in AHH activity as well as suppression of Ab responses (Holsapple *et al*., 1986). These studies suggest a possibility of Ah-dependent mechanisms for immunosuppression, but do not conclusively exclude their role in TCDD-mediated suppression of humoral immunity. These studies illustrate the controversy surrounding the role of Ah locus on TCDD-induced suppression of humoral immunity. This controversy may in part be due to the role played by accessory cells in humoral immunity.

Studies suggested that TCDD does not affect naïve T-cells but inhibits the functions of only those T cells activated *in vivo* through antigen priming (Lundberg *et al*., 1992). For example, T-cell responsiveness in the TCDD-treated conalbumin-primed mice was suppressed only in response to stimulation with conalbumin but not in response to stimulation with polyclonal T cell mitogens such as concanavalin A (Con A) or anti-CD3. The exact mechanism by which TCDD inhibits antigen-specific T cell responsiveness is not clear.

In order to gain an understanding of TCDD-induced immunosuppression, we wish to examine the effects of TCDD on the thymus and delineate the mechanisms by which TCDD causes thymic atrophy.

Specific Aims:

TCDD is well known for its immunotoxic effects, particularly on the thymus. The exact mechanism by which it induces thymic atrophy is not known. Several attempts have been made to elucidate the mechanisms responsible for TCDD-induced thymic atrophy. Some studies have suggested that TCDD, rather than acting directly on thymocytes, acts through a receptor in the thymic epithelial cells (Greenlee *et al*., 1984). In contrast, McConkey *et al*. (1988), suggested that TCDD may kill immature thymocytes by initiating apoptosis. These studies were based on addition of TCDD to *in vitro* thymocyte cultures. Several chemicals have been shown to induce apoptosis in thymocytes (Comment *et al*., 1992). These studies however, failed to demonstrate loss of viability following addition of TCDD to in vitro thymocyte cultures and therefore were unable to confirm the previous studies of McConkey *et al*. (1988). Whether TCDD-mediated thymic atrophy *in vivo* is triggered by apoptosis remains a possibility requiring further consideration. The thymus is an important organ for the maturation of T cells and therefore important in T cell mediated immunity to xenobiotics. Studies focusing on cellular mechanisms by which TCDD mediates thymic atrophy may give us insights into the complex actions that take place in the thymus and also lead to understanding its role in cancer. In the current study, the central hypothesis that apoptosis plays a key role in TCDD-induced thymic atrophy and immunotoxicity, was tested.

The specific aims of the research were to determine:

1. Whether TCDD induces apoptosis in thymocytes in vivo.
2. The phenotypic alterations caused by TCDD in thymocytes *in vivo* and its correlation with apoptosis.
3. Whether *lpr* and *gld* mice, which are defective in Fas and FasL expression, respectively, are more resistant to TCDD-mediated immunotoxicity.
4. To further characterize the mechanisms of TCDD-induced apoptosis.