PART I

INTRODUCTION
1.1 Immune system as a target for toxicity

The immune system has been documented as a target for numerous agents following either acute or chronic exposures. Radiation, environmental chemicals, contaminants, therapeutics, and street drugs are some of the agents that can alter and impair immune function. The exposure levels that impact immune function are often substantially lower than those causing overt toxic effects to other organs.

The immune system is composed of several different components that act in concert to protect against antigens or infectious agents. Identification of the targets of immunotoxic agents is important in assessing the risks of exposure. As the functional immune cells arise from hematopoietic precursors in the bone marrow of the adult, or the liver of the developing fetus, evaluation of the effects on these targets as well as the peripheral immune organs by toxic agents in laboratory testing is key to understanding the potential effects that may be noted in the human population after exposure.

Concern regarding these immunotoxic agents stems from the growing support that those compounds targeting the thymus or thymocytes may increase risk of immune dysregulation (Shuurman et al, 1992; Holladay, 1999). Thymic effects can involve the thymocytes directly, the stromal or epithelial cells, as well as the dendritic or antigen presenting cells. Because appropriate function of all these cell populations is necessary for the thymocyte maturation and selection process, effects on any population may be significant. Suggestions that the consequences of exposure to chemical or physical agents may not only decrease the thymic output of the new T cell repertoire, but also
create an unwanted repertoire of autoreactive cells, must be addressed to understand those factors that may be leading to increase in the incidence of immune system diseases. Support for diethylstilbestrol's (DES) or 2,3,7,8-tetrachlorodibenzo-p-dioxin's (TCDD) potential to elicit or promote an environment for immune dysregulation is also offered in a presentation by Silverstone et al, (1998), of an experiment using the NZB x SWR (SNF₁) mouse to investigate increases in autoimmune nephritis demonstrated by this mouse strain. The males usually do not develop significant disease prior to one year of age; however the administration of either estradiol or TCDD monthly to adult male mice caused significant glomerular damage within the first six months of life. Perinatal exposures to TCDD and DES also induced similar disease states in males between 5 and 10 months of age. The SNF₁ mouse is considered to have a less responsive TCDD genotype, suggesting that mouse strains sensitive to TCDD may respond at even lower levels than the 5-80 µg/kg range utilized in this experiment.

Estrogens, such as DES, are implicated in alterations of extrathymic T cell differentiation leading to potentially autoreactive cells (Okuyama et al, 1992). Administration of 1 mg of estrogen in male mice resulted in concurrently increased mononuclear cells found in the liver and a marked decrease in the number of thymocytes noted 10 days after injection. The mononuclear cells identified from the liver showed an increase in the αβT cells with intermediate T cell receptors (TcR) including the forbidden oligoclones, identified as extrathymic αβT cells. This activation and proliferation of extrathymic T cells is concerning because these cells with the unique characteristic of autoreactivity are implicated in autoimmune disease.
The thymic effects of TCDD and DES are remarkably similar. Decreased thymic cellularity, alteration of thymocyte phenotype, and thymic atrophy are noted. In addition, alterations in the fetal liver and the neonatal or adult bone marrow lymphoid progenitors have been described. Identification of lymphoid cell markers allows further characterization of the toxic effects of these agents on the immune system.

1.2 Human exposure to TCDD and DES

Human exposures to TCDD and related halogenated aromatic hydrocarbons (HAH) have been documented and include acute accidental exposures and chronic occupational exposures. Low level chronic exposures to this ubiquitous environmental contaminant are of increasing concern. In these circumstances, the levels of TCDD have been evaluated from blood samples and are believed to correlate with total body burden. Analysis of human tissues suggests that in some forms of dioxins and related compounds, substantial liver retention as well as lipid sequestration exists and that blood values may underestimate the total body burden. In addition, TCDD and many of its related congeners act via the aryl hydrocarbon receptor (AhR), and the use of toxic equivalency factors (TEQ) to estimate total effects of all exposures has been supported (DeVito et al, 1995). The half life of TCDD in the human is lengthy at approximately 7 years (Flesch-Janys et.al, 1996), compared to the markedly shortened half life noted in many of the laboratory animals used for experimental investigation. Concern that accumulation of not only dioxin, but also its related congeners, from human fetal exposure onward suggests that the present level of chronic daily exposure via ingestion may be one fifth (1/5) of that required to cause reproductive toxicity as based on the rodent model (Maruyama et al, 2003).
In the well-documented human exposure in Seveso, Italy, the primary effect noted after TCDD exposure was chloracne. An inverse relationship between the serum levels of immunoglobulin, IgA, and serum TCDD levels was noted in exposed individuals (Baccarelli et al, 2002). In chronic occupational exposures, the three measures significantly altered in the workers compared to the controls were increased antinuclear antibodies in 8 workers, increased immune complexes (11 in workers, 3 in controls), and lowered natural killer cell numbers as identified by monoclonal antibody Leu-7 (Sweeney and Mocarelli, 2000). In another study (Jung et al, 1998), a group of 29 occupationally exposed individuals were matched with 28 non-exposed and a variety of immunologic parameters were examined. No correlation between rates of infection or responses to vaccination and exposure was found. The \textit{in vitro} experiments demonstrated that the chromate resistance of lymphocytes stimulated by phytohemagglutinin of highly exposed persons was significantly lower than that for the control group, indicating that the function of lymphocytes can be stressed and possibly impaired by high exposure to polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).

A study from the Netherlands investigated the effects of background levels of dioxins and polychlorinated biphenyls (PCBs) exposure on the fetus and neonate. Maternal blood and breast milk levels of these chemicals were determined and infants followed to 18 months of age. Of all parameters examined, no relationship between pre- and postnatal PCB/dioxin exposure and upper/lower respiratory tract symptoms or humoral antibody production was found. However, in higher PCB/dioxin exposure, an increase was noted in TcRγδT cells at birth, and an increase in total number of T cells and the number of CD8+ (cytotoxic), TcRαβ+ and TcRγδ+ T cells present at 18 months.
of age. The higher prenatal as well as postnatal PCB/dioxin exposure was associated with lower monocyte and granulocyte counts at 3 months of age. The study suggests that background levels of PCB/dioxin exposure influences the human fetal and neonatal immune system (Weisglas-Kuperus et al, 1995).

In a review of prior studies (Kimbrough and Krouskas, 2001), the effects of PCBs, dioxins, and dibenzofurans on children were discussed. Specifically, the potential alterations on immune function, thyroid function, and birth weights were evaluated. Although trends were mentioned, the conclusions of most studies were dismissed as all results for control and exposed subjects fell within reference intervals for normal or healthy children.

While human exposure to TCDD are accidental, occupational, or inadvertent, DES has been widely used a therapeutic drug to prevent miscarriage after evidence of the role of hormones in maintaining pregnancy was demonstrated decades ago (reviewed by Bamigboye and Morris, 2003). This treatment of pregnant women resulted in an increase in the rate of miscarriage and preterm birth. Some exposed female offspring have increases in cancer of the genital tract and other forms of cancer, while rates of testicular abnormalities were higher in exposed than unexposed males. In utero exposure of the female also leads to increased primary infertility and adenosis of the vagina and cervix. The link between in utero treatment of females with DES and increased incidence of autoimmune disease has been examined in a small study. Demonstration that the prenatal exposure to DES led to an increased incidence of 50% over non-exposed cohorts was published in a retrospective study (Noller et al, 1988). In a more recent study (Burke et al, 2001), female subjects were matched by age and menstrual cycle and evaluation of T
cell mediated immune response made. The incorporation of tritiated thymidine by T cells from in utero DES exposed women was increased compared to that of untreated women in response to concanavalin A, phytohemagglutinin, as well as in response to endogenous interleukin-2. This small study demonstrates an alteration in T-cell mediated immunity; however, how this relates to possible pathogenesis in autoimmune disease is not yet understood. Another study from the Netherlands also suggested that daughters of women treated with DES during pregnancy have an increased risk of developing immune related diseases (Vingerhoets et al, 1998). Both men and women who were exposed to DES in utero responded to a questionnaire concerning their health status in a 1998 survey (Wingard and Turiel). Increased reports of respiratory tract infections, asthma, lupus, and arthritis were noted among the respondents, suggesting that DES exposure may have altered or impaired their immune systems. Additionally, in adult men, DES has been used as an adjunct or primary therapy in the treatment of prostatic carcinoma (Chodak et al, 2002). Hormone replacement therapy in postmenopausal women is increasing in order to gain the positive effects of estrogen on multiple systems, including the urinary tract, cardiovascular system, and as a protectant against osteoporosis (Butler et al, 1995; Cardozo and Kelleher, 1995). These deliberate therapeutic exposures to estrogens create an increase concern for the possible immunotoxic effects it may have on the human population.

Other compounds acting as endocrine disrupters via the estrogen receptors have been implicated in immune system disease, including bisphenol-A, phthalate, nonylphenol, polychlorinated biphenyls, and dioxins. Additionally, naturally occurring plant estrogens and mycotoxins, such as zearalenone, may have an impact on estrogen
responsive tissues, including the immune system. These compounds are found in the environment as well as the diet, leading to chronic human and animal exposure. While most of these chemicals are less potent than estrogen or diethylstilbestrol, they are also chemically stable and able to bioaccumulate in adipose tissue (Ahmed, 2000).

1.3 Laboratory modeling

The mouse has been used as an experimental model for toxicologic investigations for many years. The National Toxicology Program in cooperation with many other laboratories developed testing protocols to evaluate immunotoxicants. The application of these protocols to developmental studies must be adjusted for the immaturity of the fetal immune system. However, organ weights and cellularity, and cell surface markers can be used in evaluating late gestation feti, as well as postnatal and adult testing of gestationally exposed subjects for the developmental evaluation of an immunotoxicant (Holladay and Blaylock, 2002). Although numerous studies in laboratory animals have been conducted on dioxin and dioxin-like chemicals, the application of the results to potential human effects must be made with caution. Humans are exposed to multiple chemicals over time, while experimental animals typically live in a controlled environment with well-defined exposures to agents (DeVito et al, 1995). In addition, the rate of removal of different agents from the body varies between humans and laboratory rodents. Further, wide variations to the effects of a specific dosage of dioxin are seen within laboratory species, and even strains of species. Resistance or susceptibility to dioxin has not been linked to the binding affinity of dioxin to the receptor in a cross comparison of species. For example, while the dioxin receptor binding takes place at lower concentrations in the mouse than in human tissues in vitro, inhibition of thymocyte proliferation occurs at the
same concentration for both species in cell cultures. Other effects can be seen that vary in orders of magnitude in concentration differences, with human tissue effects seen at similar or even lower concentrations, as found with lymphocyte proliferation.

1.4 Ontogeny of the thymus

One of the vital components of the immune system are the lymphoid cells. These cells originate from pluripotent stem cells in the bone marrow or fetal liver and differentiate through the common lymphoid progenitor, eventually becoming committed to either B cell or T cell lineages. The B cells are responsible for humoral immunity, that is, the production of antibodies in response to antigen. The T cells are, in fact, more diverse in that they develop into distinct subtypes depending on their function and expression of cell surface markers. Progenitors of T cells migrate to the thymus where they mature and differentiate via processes of positive and negative selection into a variety of mature T cells including T helper cells (CD3⁺CD4⁺CD8⁻TcRαβ), cytotoxic T cells (CD3⁺CD4⁻CD8⁺TcRαβ) and a final, small group of T cells with CD3⁺CD4⁻CD8⁻TcRγδ surface marker expression (Elgert, 1996).

Early organogenesis of the murine thymus occurs between gestational days 9.5 and 11.5, and primarily consists of the interactions between the thymic epithelium and mesenchyme to create the thymic rudiment (Manley, 2000). When the early epithelial rudiment is encapsulated by the condensing mesenchymal cells, signals promoting growth are released. Hematopoietic cells arrive shortly after the formation of the rudiment on days 11 to 11.5. The first wave of hematopoietic cells on days 11-12 may migrate from the aorta-gonad-mesonephros or from the fetal liver. For the remainder of gestation the thymus colonizing precursors arise from the fetal liver. Between days 17
and birth in murine gestation, the fetal marrow becomes the primary site of hematopoiesis (Carlyle and Zuniga-Pflucker, 1998). Recent investigations demonstrate that the majority of progenitors in murine fetal blood from gestational days 12 to 13 are pre-thymocyte precursors. Their numbers are diminished after gestational day 15. These same cells are found in athymic nude fetal mice in numbers similar to those seen in wild type fetal mice (Ikawa et al, 2004). These cells in the fetal blood are closely related to the pre-T cells in the fetal liver and earliest fetal thymus in that they have the same cell surface markers as those seen in the fetal liver and the earliest cells in the fetal thymus (Lin−c-kit+IL7R+). Their lineage and stage specific genes are similar in gene expression profiles of those T cell progenitors in the fetal thymus and liver; a single T cell progenitor from the fetal blood is able to produce thousands of T cell precursors prior to TcRβ chain gene rearrangement, and these fetal blood pre-T cells are able to differentiate into natural killer (NK) and dendritic cells (DC). Their presence in athymic mice supports the possibility that these early T cell precursors may be responsible for seeding the other tissues, such as the skin. The study also demonstrated that fetal thymic pre-T cells are different from those of the adult in that fetal pre-T cells are IL7R+ while adult thymic pre-T cells are IL7R−, supporting the notion that fetal hematopoiesis differs from that of the adult. The presence of all three cell types, hematopoietic, mesenchymal, and epithelial, is required for the proper patterning and epithelial differentiation in late stage organogenesis.

The mesenchymal cell derived from the neural crest appears to be key to normal thymocyte development since experiments in which the fetal thymic mesenchyme is removed results in lymphoid cells that fail to develop properly and are blocked at the
CD4 CD8\(^{+}\) double negative stage (Owen et al., 2000). As the thymus is poorly vascularized early in development, prothymocytes must migrate through the mesenchymal capsule to gain entry to the cortex. In experiments in which the capsule is removed by microdissection on gestational day 12 and thymic lobes then supported in vitro, alterations in thymic size were observed in addition to the altered thymocyte maturation. After 10 days of culture, the lobes without mesenchyme were dramatically smaller than those with associated mesenchyme. The thymic mesenchymal cells are also responsible for the production of the extracellular matrix. This matrix material consists of fibronectin, proteoglycans, and laminin arranged in an orderly fashion. Thymocytes express integrins capable of interacting with the extracellular matrix. In particular, IL-7, an important cytokine in thymocyte development, is known to bind to proteoglycans and in this way may be presented to the developing thymocytes via the extracellular matrix. As the thymus continues development, the mesenchyme forms the septae and lobules of the thymus and maintains an intimate relationship with both the thymic epithelium and the maturing thymocytes (Suniara et al, 2000).

All lymphoid progenitor cells are derived from pluripotent stem cells found in the fetal liver and adult bone marrow. Those classified as early lymphoid progenitors have lost some of their capacity to form other types of blood cells. The T lymphocytes are no longer considered early cells once they have completed gene rearrangement specifying either the \(\beta\) or \(\gamma\) chain of the T cell antigen receptor (Shortman and Wu, 1996). Distinct differences in the fetal and adult hematopoietic stem cells which populate the thymus were noted. The first wave of fetal T cells give rise to the Thy-1\(^{+}\) dendritic cells found in the skin. Both the hematopoietic stem cell from the fetal liver and processing through the
fetal thymus are necessary for the production of these cells. Studies using adult hematopoietic stem cells to repopulate the fetal thymus failed to yield the specific epidermal dendritic cells produced normally by the fetal thymus (Shortman and Wu, 1996). Further evaluation of hematopoietic cells have permitted the identification of thymocyte progenitors by cell surface markers and determination of the presence, absence, or relative expression levels (low, intermediate, or high) of these markers as the thymocytes progress. These markers include CD44, CD25, c-kit, HSA, the T cell receptor (CD3), Thy-1, and the CD4 and CD8 markers identifying T-helper cells and cytotoxic T-cells (Zuniga-Pflucker and Lenardo, 1996).

Characterization of the fetal thymus progenitor cells and comparison with those present in the fetal liver has been performed. By flow cytometric evaluation, markers on fetal thymus harvested on gestational day 12 and cultured for 10 days revealed that progenitors restricted to thymocytes, B lymphocytes and myeloid lineages are present, but no pluripotent stem cells are found in the fetal thymus. The fetal liver possesses both lineage-restricted and pluripotent stem cells in numbers much higher than the fetal thymus. The fetal liver contains twenty times more of the pre-T cell committed progenitors than are found in the fetal thymus. The pre-T cells of the fetal liver are also present in the fetal blood. However, these liver pre-T cells are distinctly different from those in the fetal blood on gestational day 15.5 which are Thy-1^+c-kit low (Kawamoto et al, 1998). Also, the Thy-1 (dull) γδT cells have been determined to be a distinct T cell subset in the adult mouse that contain TcR gene rearrangements typical of those seen in the fetal thymus. In experiments, few of these γδT cells develop in the adult mouse after radiation exposure if reconstitution is performed with either adult bone marrow or fetal
liver hematopoietic tissue as the source. However, if fetal thymi are grafted into adult recipients, normal numbers of Thy-1 (dull) γδT cells are present in the grafts. These T cells are of graft origin even when most of the other thymocytes and thymocyte precursors are of recipient origin (Grigoriadou et al, 2003). In addition to intrathymic development of T lymphocytes, extrathymic T cell development has been studied. The liver continues to contain c-kit+ stem cells which have the capacity to differentiate into the T cells, NK cells, or even granulocytic cells after birth. The extrathymic T cells are relatively low in number initially. However, their number increases with age and with different physiologic conditions, including stress, cancer, pregnancy, autoimmune disease, and chronic graft versus host disease as examples. The autoreactive forbidden clones of T cells are found within this extrathymic subset of thymocytes, and therefore overactivation or alteration of their activity may predispose the onset of autoimmune disease (Abo, 2001).

1.5 The Estrogen Receptor

The estrogen receptor (ER) has been identified in two subtypes, ERα and ERβ, both located intracellularly functioning as ligand activated transcription factors. Estrogen receptors are present in diverse tissue types and affect growth, differentiation, and proliferation. The ligand-receptor complex with its coregulatory proteins then effect gene transcription on estrogen responsive elements of the nuclear DNA. A number of structurally diverse natural or synthetic compounds act on the estrogen receptors (Katzenellenbogen et al, 1999). Natural estrogens, such as the endogenous estradiol, as well as zearalenone, coumestrol, and enterolactone, all act via the estrogen receptor as do the synthetic estrogens, diethylstilbestrol, bis-phenol A, Kepone, and o,p'-DDT. Their
affinity for the receptor varies as does their activity, determined by the ER to which they are bound.

The receptors ERα and ERβ are both present on the thymocytes and thymic stromal cells, with most ER expression localized to the double positive CD4^+CD8^+ thymocytes (Mor et al, 2001). In this in vivo study, ovariectomized female rats were treated with estrogen or saline. Estrogen treatment decreased thymus size and increased FasL expression in the thymus of these animals.

Attempts to define the role of the estrogen receptors in normal immune development have been made. Erlandsson et al (2001) used estrogen receptor knockout mice, selecting for single knockout ERα (ERKO) or ERβ (BERKO), and double knockout ER (DERKO) mice lacking both receptors. It was noted that in the ERKO male mice, both the thymus and spleen were hypoplastic. A higher frequency of immature double positive CD4^+CD8^+ cells was found in the ERKO mice compared with ERα^+ mice. Other appreciable differences were found in BERKO mice as estradiol administration to oophorectomized females resulted in similar degrees of thymic atrophy, but only slightly decreased cortical involution, when compared to wild type mice. These findings suggest that ERα may be necessary for the development and full functioning of the immune system in males, while ERβ is required for estrogen mediated thymocyte phenotypic alterations and thymic atrophy.

In another study by Li et al (2002), the role of estrogen via its activity on the estrogen receptor was examined. In those knockout mice lacking aromatase, an enzyme necessary for estrogen synthesis, thymocyte development was impeded and reduced
cellularity was noted relative to wild type mice. Impaired progression of cells to the double positive state as well as diminished double positive cell proliferation were noted. These maturation arrests were postulated to possibly contribute to the decrease in thymic cellularity found in these knockout mice. Estrogen thus appears to support thymocyte development and maturation and affects many stages via regulatory pathways.

1.6 The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is a cytosolic protein with co-chaperone proteins that dissociate when ligand binding occurs. The receptor-ligand complex translocates to the nucleus where it binds with the aryl hydrocarbon nuclear translocator protein (ARNT). This ligand-AhR-ARNT complex then acts by binding to xenobiotic responsive elements and turns on the transcription of targeted genes. Some of the identified genes encode enzymes that may break down ligands or convert them to more toxic metabolites. Conversion of some AhR ligands increases their toxicity by creating mutagenic derivatives (Hankinson, 1995). The AhR is remarkably promiscuous and binds to a variety of compounds, few endogenous, but numerous exogenous, both naturally occurring and man-made. Some of the endogenous ligands include tryptophan, indole metabolites, bilirubin, indirubin, and arachadonic acid metabolites (Denison and Nagy, 2003). However they are present in such low levels in the normal physiologic model, ascertaining their role as AhR ligands is difficult (Elferink, 2003). Other naturally occurring ligand are derived from fruits, vegetables, herbs, and flavonoids (Zhang et al., 2003). Of those evaluated in human cell lines, agonistic activity of the AhR was found to be between 20-60% of that produced by TCDD. Antagonistic activity of the AhR by
these naturally occurring ligands could be overcome by dilution resulting in AhR
agonistic activity to levels similar to TCDD (Jeuken et al, 2003).

The natural function of the AhR has been a topic of interest for some years, and
the production of AhR −/− mice permitted further discovery of the AhR function in
development. AhR −/− mice had decreased cellularity of the spleen and lymph nodes as
well as reduced liver size. Bile duct fibrosis was noted in these mice as well. When
exposed to TCDD, these mice did not display the dioxin-mediated induction of genes
encoding enzymes that is typically seen with AhR ++ strains. These data suggest that
the AhR plays a role in the normal development of the liver and immune system
(Fernandez-Salguero et al, 1995)

TCDD is not genotoxic, but activates the AhR/ARNT complex leading to
transcriptional changes by the cell. Suggestion has been made that the AhR is a
pacemaker for the cell, leading to cell cycle progression depending on the ligand
activation or unbonded form, and potentially causing cell cycle arrest with TCDD
administration by altering gene expression, including increased expression of cyclin-
dependent kinase 2 inhibitor, p27kip1 (Elferink, 2003).

Evaluation of two strains of AhR deficient mice demonstrated that the AhR is not
necessary for cell mediated immunity (Vorderstrasse et al, 2001). In adult mice, both
strains were able to mount appropriate responses to a challenge of tumor cells, or respond
with antibody production to sheep red blood cells. When treated with TCDD, these AhR
deficient mice were unaffected, and antibody production or cell mediated immunity were
both similar to controls. In animals with AhR ++ genotype, TCDD treatment
significantly suppressed cell mediated immunity and humoral response. Interestingly,
those animals cross bred with an AhR +/- genotype had variable responses in the cytotoxic T lymphocyte assay, and experienced a suppression in antibody production, however this was considerably less than the homozygous AhR +/- strains of mice.

The use of AhR +/+ and AhR +/- fetal thymic organ culture (FTOC) supports the activity of TCDD in the fetal thymus via the AhR. In those AhR -/- FTOC, no effect of TCDD was seen compared to untreated AhR -/- cultures. However, overall, the AhR -/- untreated cultures, when compared to AhR +/- control cultures, had lower cellularity suggesting that the AhR is important in normal thymic development. In addition, in the AhR -/- cultures, an increase of double negative immature cells with an $\alpha\beta$TcR was seen. Comparisons of neonatal AhR +/- and AhR -/- mice and weekly evaluation showed that the AhR -/- mice have 75% fewer B cells and T cells in the spleen and peripheral lymph nodes at 2 to 3 weeks of age. However, these increased to normal values at week 10. This suggests an active role of the AhR in lymphoid seeding or proliferation of B and T cells (Hundeiker et al, 1999).

Surprisingly, TCDD as an AhR agonist leads to inhibition of 17$\beta$-estradiol changes noted in responsive tissues. Normally the administration of 17$\beta$-estradiol, which acts on the estrogen receptor (ER), leads to increases in uterine weights, induces cellular proliferation, affects progesterone receptor binding activity, and epidermal growth factor receptor binding and receptor mRNA levels in cells susceptible to the estrogenic compounds (Safe, 2001). Co-administration of TCDD with 17$\beta$-estradiol inhibits these responses. In a study to understand the mechanism of this receptor cross-talk in rodent uterine and mammary tumors and human breast cancer cell lines, TCDD suppressed 17$\beta$-estradiol-induced responses. In a number of human breast cancer cell lines, TCDD was
determined to induce specific proteasomes which degrade estrogen receptor α, and the use of proteasome inhibitors could restore the estrogenic effects of 17β-estradiol (Wormke et al, 2003).

1.7 Effects of DES and TCDD on the immune system

TCDD has been investigated for its effects on the immune system for decades. In early experiments, Vos et al (1973) noted species differences in sensitivity and responses to the toxicant. In rats, oral dosing of 5 µg/kg once weekly for 6 weeks resulted in decreased body weights in addition to relative and absolute decreases in thymic weights and slight to moderate atrophy of the thymus. The rats demonstrated no alterations in cell mediated immunity. In the same experiment, guinea pigs receiving a weekly oral dose of 1 µg/kg of TCDD for eight weeks either died or were euthanized when moribund by day 32 prior to the completion of the experiment. Severe weight loss was noted in these animals as well as lymphopenia and depletion of the lymphoid organs, particularly the thymus. Severe atrophy of the thymic cortex was accompanied by the destruction of lymphocytes and increased nuclear debris. The phagocytic activity of the macrophages lead to the "starry sky" appearance of these tissues. For those guinea pigs in the lower dosage groups, cell mediated immunity was significantly depressed at the 0.2 and 0.04 µg/kg dose levels. Mice receiving chronic administration of TCDD also had significantly reduced thymic weights at the 5 and 25 µg/kg dose levels. In this same experiment, donor mice were administered vehicle or 0.2, 0.1, 5.0, or 25 µg/kg TCDD. After sacrifice, splenic cell suspensions were made and administered to recipient mice to measure graft-versus-host response. The graft-versus-host response was significantly
suppressed at the 5 µg/kg dose, and was only 62% of the control level in the 1 µg/kg dose group.

In 1974, Vos et al. examined the effects of short term oral administration of TCDD to C57Bl/6 mice. In this experiment, 0, 0.2, 1, 5.0 or 25 µg/kg doses of TCDD were given once a week for 2 to 6 weeks. Interestingly, at the no-effect level for total body weight (0.2µg/kg), severe lesions in the thymus and liver were still noted. Decreased serum gammaglobulin levels were also seen at the 5 and 25 µg/kg levels, suggesting suppression of humoral immunity.

Both direct and indirect effects of TCDD on immune function have been suggested. Splenocytes were harvested from C3H male mice and exposed in vitro with increasing molar concentrations to each of four known immunotoxicants and then the gene expression of pro-inflammatory cytokines assessed. TCDD increased the gene expression of interferon-γ and tumor necrosis factor-α, but suppressed the expression of interleukin-1 (Ho-Jun et al, 2002). These data support the hypothesis that the effects of TCDD may be indirectly related to alteration in those cytokines which regulate the inflammatory response. Similarly, TCDD was found to suppress both humoral and cell-mediated responses to a non-lethal influenza virus without affecting the cytolytic activity of T-cells recovered from the lungs of C57Bl/6 mice experimentally infected (Warren et al, 2000). TCDD was administered by a single oral dose of either 1, 5, 10 µg/kg one day prior to intranasal infection. Dose-responsive mortality was exhibited in the TCDD treated groups, while no mortality was seen in the control animals. Interferon-γ was noted to be ten fold higher in the bronchoalveolar lavage fluid recovered from TCDD exposed mice. This increase in interferon-γ may lead to excessive inflammation,
activation of macrophages, and recruitment of neutrophils leading to excess tissue damage. In a 1990 study (House et al), the effect of TCDD administered 7-10 days prior to challenge with various pathogens resulted in a significant decrease in antibody forming lymphocytes. This dose-dependent response was noted for both T-dependent and T-independent antigens. However, no effect was found on natural killer cell function, interferon production, or macrophage function. Although an increase in susceptibility to influenza virus was noted, no changes were noted in control and treated animals in response to bacterial infection with *Listeria monocytogenes*. Burleson et al (1996) sought to find the No Observable Adverse Effect Level (NOAEL) of TCDD when administered to mice 7 days prior to influenza virus challenge. Increased mortality in mice was seen after a single doses of 0.1, 0.05, and 0.01 µg/kg TCDD prior to infection with Hong Kong influenza virus. Single doses of 0.005 and 0.001 µg/kg TCDD had no effect on influenza-induced mortality. Interestingly, the increase in mortality at 0.01 µg/kg did not correspond to a decrease in thymic weight that is characteristic of TCDD. Decreases in thymic weight associated with Hong Kong Influenza virus infection in control and treated animals were similar. No additive or synergistic influence was noted with TCDD treatment.

The production of antibodies, cytokines and T-cell growth factors was also investigated after TCDD exposure (Nohara et al, 2002). Oral administration of 5 or 20 µg/kg TCDD and injection with ovalbumin (OVA) was concurrently followed by re-injection with OVA 21 days later. In the TCDD exposed animals, antibody production was reduced, and thymic weights and cellularity reduced by the 20 µg/kg dose. However, splenic weights and cellularity were not altered by either dose. An increase in
interferon-γ production by the TCDD exposed animals was noted. In addition, a reduction of IL-4 and IL-5 was noted in the TCDD exposed animals. Both of these cytokines affect the functions of B cells, and therefore their diminished production by T cells may result in the altered humoral response of the treated mice.

Evaluation of dietary phytoestrogens that act via the estrogen receptor and their effects on the immune response has been performed (Curran et al, 2004). Diets rich in soy estrogens were fed to mice of wild type and ERα-deficient mice, and the mice then infected with *Mycobacterium avium*. Alterations in splenic interferon gamma and interleukin-18 were noted in the wild type mice, as well as an increase of IL-12 tissue levels in the ER-deficient mice. This research concluded that the presence of the estrogen receptor and dietary phytoestrogens can influence the production of cytokines in response to a chronic bacterial infection.

DES has demonstrated marked effects in fetal, neonatal, and adult exposures. However, interest in its effects on the aged subject is investigated as its use in the elderly human population increases. Smith and Holladay (1997) exposed senescent (21 months of age) C57Bl/6 female mice to 1.5 or 6.0 mg/kg DES for five consecutive days via intraperitoneal injection. Comparison of the treated mice with control animals showed a significant thymic hypocellularity and an alteration in thymocyte maturity, including a decrease in the double positive CD4⁺CD8⁺ population and a concomitant increase in the double negative CD4⁺CD8⁻ cells. Bone marrow cellularity was not affected by DES, though alterations in the percentages of common leukocyte, macrophage, and B lymphocyte precursors were observed. In the spleen, total numbers of cells of the T
lymphocyte lineage were reduced. These alterations appeared more severe in the geriatric model than those seen in young adult mice.

A study by Utsuyama et al (2002) suggests that the age and gender of the subject can play a substantial role in the response to exogenous estrogen exposure. This study supports the notion that investigations of the compounds acting as estrogens need to be completed over a range of ages to project potential times of concern in human development and aging.

1.8 Targets of DES and TCDD: the thymus and its hematopoietic precursors

One of the targets for TCDD's effects is the antigen-presenting activities of the dendritic cells (Vorderstrasse et al, 2003; Vorderstrasse and Kerkvliet, 2001). In 2001, mice were exposed via oral gavage at a dose of 15 µg/kg and surface accessory molecules of the dendritic cells evaluated for alterations. Alterations, both increases and decreases, of varying molecules were noted. Co-culture of these dendritic cells with T cells caused an increase in the production of IL-2 and interferon gamma by the T cells. However the total number of dendritic cells was decreased in the treated mice. In the absence of an antigen, TCDD was also found to provide an activation stimulus to dendritic cells which may lead to their premature deletion. Survival of the dendritic cells has been shown to influence the strength and duration of the immune response. The results obtained suggest another avenue of influence TCDD has on the immune system.

In the 2003 study, using C57Bl/6 and BALBc mice, TCDD was administered at a dose of 15 µg/kg via oral gavage. In these treated animals, activation of dendritic cells was noted without antigenic stimulation. Although TCDD did not suppress the ability of the dendritic cells to process and present antigen, activation of these cells reduced their
ability to phagocytize antigen and may impede their ability to provide activation signals to the T cells. This failure of signaling may alter the survival of the dendritic cells, the T cells, or both, and might lead to the dysregulation of the immune response.

Conflicting reports on the effects of TCDD on the thymic stroma or epithelial cells have been published. Some researchers describe these thymic cell populations as sensitive and contributory to the alterations present in treated animals (Kremer et al, 1994). Using whole fetal thymic cultures and depleting them of developing T cells by treatment with deoxyguanosine, the remaining T-cell free thymic lobes were exposed to various concentrations of either TCDD or 3,3',4,4'-tetrachlorobiphenyl (TCB) and then replenished with thymocytes from untreated or treated fetal mice and the thymocytes permitted to develop. TCDD treatment of the stroma resulted in lower overall thymocyte numbers when compared to the untreated stroma, but did not prevent maturation. Furthermore, TCDD treated thymocytes, when placed in culture with untreated stroma, differentiated into mature forms, supporting the hypothesis that TCDD's effects on the non-lymphoid compartment of the thymus contributes to the atrophy noted.

Others suggest that targeting of these stromal or epithelial cells by TCDD occurs, but is not related to thymic hypopcellularity (Staples et al, 1998). Congenic mice with either TCDD-responsive (AhR+/+) or non-responsive (AhR-/−) alleles were used to create chimeras. Mice of both genotypes were irradiated to remove hematopoietic cells. Reconstitution of these mice was made with AhR−/− mice receiving bone marrow from AhR+/+ donors, while AhR+/+ mice received bone marrow from AhR−/− donors. In this way the effects of TCDD on either the stromal or hematopoietic compartments could be isolated in an in vivo system. When mice with AhR−/− hematopoietic elements and
AhR+/+ stromal elements received TCDD after reconstitution, no indication of TCDD treatment was seen. No phenotypic alterations of thymocytes were noted in these animals. However, those chimeric mice with sensitive hematopoietic elements and non-responsive stromal elements had significant declines in thymic weight and cellularity after TCDD treatment. These effects were similar to those seen in the mice with AhR+/+ hematopoietic and stromal elements. The thymocytes from these hematopoietic sensitive animals also had phenotypic changes with a decrease in the double positive population (CD4⁺CD8⁺) and an increase in the double negative (CD4⁻CD8⁻) and single positive (CD4⁻CD8⁺) cells when compared with control percentages.

A similar more recent study (Tomita et al, 2003) involved the use of transgenic mice with the disruption of the Arnt gene in T cells only. Such mice had normal development and showed no changes in mortality or growth. Development of the thymus and thymocyte population in Arnt deficient mice were similar to wild type mice. When exposed to TCDD, the transgenic mice lacking Arnt protein were resistant to its effects and were comparable to control animals in cellularity and thymocyte phenotype distribution. In addition, T cell-specific Arnt-disrupted and epithelial cell-specific Arnt-disrupted fetal thymic organ cultures were used to examine the role of thymic epithelial cells in mediating TCDD's effects. Flow cytometric analysis demonstrated that those cultures with normal Arnt producing T cells were susceptible to TCDD even if the epithelial stroma was Arnt-disrupted. Cultures with Arnt disrupted T cells and normal Arnt producing epithelial cells were resistant to TCDD effects. This study demonstrated that the T cell appears to be the primary target of TCDD in the thymus contributing to thymic involution, while the role of the stromal cells is minimal.
Studies involving human thymic epithelial cell cultures exposed to TCDD were found to alter terminal differentiation with an overall diminished cell number (Riecke et al, 2003). These changes occurred at concentrations as low as 0.01 nM and peaked at concentrations of 1.0 nM. This lowered epithelial cell number may be attributed to the enhanced differentiation, as differentiated cells have a diminished capacity for proliferation. Adhesion molecules that mediate both cell-matrix and cell-cell interactions were also altered by TCDD treatment.

The effect of sex steroids on rat thymic epithelial cells in culture was examined to understand the mechanism of the proliferative and inhibitory effects of progesterone, androgen, and estrogen (Sakabe et al, 1994). The results suggest that the thymus develops normally at adequate sex hormone levels approached before puberty. However the increased hormone levels of puberty and pregnancy act as a mechanism to minimize thymic development or proliferation at those times. Supraphysiologic concentrations of estrogen were found to diminish thymic epithelial cell proliferation.

In a review by Seiki and Sakabe (1997), the role of estrogen on the thymus is described. Estrogen receptors are located on those thymic epithelial cells which are also positive for thymulin. Treatment with estrogen results in a marked decrease in thymulin content of the cells. Thymosin mRNA expression was also significantly decreased in thymic epithelial cell culture when those cultures were treated with estrogen. These results suggest that the action of estrogen on the thymus may be mediated indirectly through the inhibition of thymic factors production by thymic epithelial cells rather than specifically targeting the thymocytes.
Lethally irradiated mice were reconstituted with bone marrow from Rag1 -/- donors and AhR -/- donors in combination to identify the target of TCDD in the bone marrow derived cells. Antigen presenting cells (APC) and dendritic cells both originate from the bone marrow or fetal liver to populate the thymus. The bone marrow Rag 1 -/- prothymocyte cells do not develop past the double negative stage, while the AhR -/- bone marrow cells were insensitive to TCDD effects. The lethally-irradiated recipient mice were found to be resistant to the effects of TCDD, even though the dendritic cells and APC’s from the donor marrow were sensitive to TCDD. Further, a decrease in proliferation of the prothymocyte bone marrow population was noted after TCDD administration when compared to controls at multiple time points. Results identify the prothymocyte population as the effective target resulting in thymic atrophy after TCDD administration (Laiosa et al, 2003).

Investigation of the hematopoietic compartment of the fetus (fetal liver) as a target for TCDD and DES has demonstrated alterations in the prolymphoid cell population. The production of the protein terminal deoxynucleotidyl transferase (TdT - a DNA polymerase marker) is diminished with TCDD, as well as decreases in mRNA expression of TdT and recombinase activating gene-a (RAG-1) within this compartment (Frazier et al, 1994). Both TdT and RAG-1 are markers for lymphocyte progenitor cells. In an earlier study, Fine et al (1988) evaluated perinatal exposure of BALB/cGa mice of a single oral dose of 10 µg/kg of TCDD on gestational day 14. Fetal livers on gestational day 18 as well as bone marrow from postnatal days 4, 11, and 18 were evaluated for TdT biosynthesis and TdT mRNA levels. TdT synthesis was reduced more than 50% in treated fetal liver lymphoid cells on gd 18. The levels present in neonatal bone marrow
were even more markedly reduced on postnatal day 18. However, thymic TdT synthesis appeared to be unaffected on a per cell basis in the face of marked thymic atrophy and decreased thymocyte number. In 1990, Fine et al examined the ability of gestationally-exposed stem cells to reconstitute the thymus and bone marrow by treating the dam with 15 µg/kg TCDD on gestational day 14 and using gestational day 18 fetal livers or postnatal day 5 bone marrow injected into sublethally irradiated DBA/2 mice which are comparatively TCDD resistant. The TCDD exposed feti and neonates, when compared to control hematopoietic stem cell donors, had diminished capacity to reconstitute the recipient thymus. Reconstitution of the bone marrow was also impaired at a lower numbers of donor cells. In young adult mice, Fine et al (1990) also examined the relative sensitivity of the bone marrow prothymocytes and thymocytes to TCDD. Administration of doses of TCDD at 5 to 120 µg/kg resulted in a dose-dependent reduction in thymic cellularity and bone marrow cellularity, with the effective dose for thymic atrophy measured histologically as being between 10 and 20 µg/kg. Bone marrow hypocellularity was less pronounced at the same doses. However, the TdT synthesizing cell in the bone marrow of the adult mouse was more sensitive to the effects of TCDD than was the TdT producing cell in the thymus on a per cell basis. Again, when TCDD treated bone marrow donors were compared to control treated donors in their ability to reconstitute the sublethally irradiated recipient thymus, TCDD resulted in a marked decrease in thymocytes present 18 days after transplant. Another study in TCDD sensitive C57Bl/6J adult mice supports the notion that the hematopoietic precursor is a sensitive target in this strain of mice (Murante and Gasiewicz, 2000). Mice received a single dose of 30 µg/kg and their hematopoietic cells were assessed for phenotypic alterations beginning at day
one and following 31 days from treatment. Examination was made of the early hematopoietic stem cells for alterations in c-Kit and Sca-1 expression to identify the potential mechanism of TCDD on the thymus and immune function. Higher levels of c-Kit and Sca-1 are associated with cells that have thymic reconstituting abilities within the lin\(^\text{Thy-1}\text{lo}\) marrow cells. Exposure to TCDD increased the absolute number of these cells relative to control bone marrow throughout the experiment. Increases in the c-Kit\(^+\) and Sca-1\(^+\) cells were noted at very low doses of TCDD (6 µg/kg). Reasons for this increase in pluripotent cells may be that TCDD causes a maturation arrest leading to a relative increase as these cells are unable to progress through differentiation, or that TCDD may cause a proliferative response in these cells. The decrease in TdT and Rag-1 synthesis noted in previous studies supports the maturation arrest at the earlier stages and suggests that TCDD has an effect on the hematopoietic cells prior to the common lymphoid progenitor stage.

In a study using gestational DES exposure, no alterations of cells expressing CD44 (hematopoietic precursors), Mac-1 (granulocyte-macrophage lineage precursors), or CD45R (B lineage lymphocyte marker) were seen in fetal livers on gestational day 18 when compared to untreated controls. A reduction in TdT, a prothymocyte marker, was noted in the livers of the DES-treated feti (Holladay et al, 1993). In addition, experiments to assess the ability of DES-treated fetal livers to reconstitute irradiated hosts resulted in reduced ability of the DES treated cells to repopulate the thymus of the recipient. Thus, DES seems to specifically target the prothymocyte of the fetal liver.

To determine the role of estrogen on the bone marrow kinetics as it relates to loss of ovarian function and increase in osteoclastic activity, female Sprague-Dawley rats
were ovariectomized and bone marrow evaluated for osteoclastic activity and and osteoclast progenitor colony forming unit-granulocyte macrophage (CFU-GM) levels. Other ovariectomized rats acted as bone marrow donors, and the marrow treated with either estrogen alone (17β-estradiol-3-benzoate) or in combination with the antiestrogen, ICI 164,384. Estrogen treatment led to a decrease in CFU-GM following ovariectomy. Ovariectomy also led to an increase in early hematopoietic stem cells. The assays for TdT showed that estrogen treatment negatively affected growth factor induced proliferation of these early progenitors by promoting apoptosis (Shevde and Pike, 1995). This study suggests that the early hematopoietic progenitor cells are targets for estrogen.

In studies evaluating the effects of TCDD on bone marrow response, offspring demonstration of a reduction in bone marrow cellularity and anemia which was dose dependent has been shown following 5 and 15 µg/kg maternal doses. The presence of pluripotent stem cells was markedly depressed relative to controls (Boorman et al, 1982).

In adult C57Bl/6 mice, a single oral dose of 2 µg/kg TCDD was given and thymocyte subpopulations in the spleen and thymus evaluated 2 days after exposure (Kerkvliet and Brauner, 1990). The total number and percentage of double positive CD4+CD8+ thymocytes were decreased, while the percentage but not total number of double negative CD4-CD8- thymocytes was increased in the TCDD treated animals. No alterations in percentages or total number of single positive CD4+ or CD8+ cells were detected in the thymus. Splenic lymphocytes were examined after a single acute exposure range of 0.2 to 5.0 µg/kg TCDD. Of the splenic lymphocytes, an elevation of CD4-CD8+ T cells in the control animals was noted after sheep red blood cell injection for PFC assay. This same subset of splenic T cells remained unchanged in the 5 µg/kg
TCDD dose group. The acute dose of TCDD reduced the splenic anti-SRBC response by 65-80%, but did not cause detectable changes in the major splenic lymphocyte populations. These results suggest that impairment of the immune system may occur in the absence of subset changes as detected by cell surface markers.

A more recent study by Tsukumo et al (2002) suggested that instead of a maturation arrest, TCDD may accelerate the differentiation of thymocytes to the CD8+ SP stage via extracellular signal-related kinase pathways. In this study, C57Bl/6N fetal thymi were harvested at gestational day 16.5 and the organ cultures were exposed to 10 nM concentrations of TCDD dissolved in DMSO. Evaluation on days 0, 2, 4, and 6 revealed that the thymocyte populations changed dramatically by day 4 with a diminution of CD4CD8 DP cells and an increase in DN, CD4+, and CD8+ cells on a percentage of total basis. By day 6 these trends were more dramatic. In evaluation of the CD8+ cells, the number of CD3hiCD8 T cells was significantly increased in total numbers in TCDD treated thymus organ cultures on day 4 when compared to controls. The use of an inhibitor of kinase pathways, U0126, was applied to the organ cultures treated with TCDD and the increase in CD8 T cells was suppressed. The increase in CD3hiCD8 T cells was absent after cotreatment of TCDD organ cultures with U0126. These data suggest that the extracellular signal-related kinase pathway is requisite in TCDD-induced increase in the CD8 T-cell population, as well as the increase in the CD3hiCD8 T cell population seen in this experiment.

A study utilizing C57BL/6 donor mice which were either AhR +/- or AhR -/- examined the effect of TCDD on the graft-versus-host response to determine if the AhR was required for TCDD activity. Irradiated recipient mice received bone marrow from
either AhR+/+ or AhR -/- donors and then were challenged. No difference was noted in the AhR -/- donor cell recipients. Animals receiving bone marrow from these AhR -/- demonstrated the same level of cytotoxic activity measured in the spleen ten days after administration whether treated with vehicle alone or with TCDD. Recipients of AhR +/- cells demonstrated a marked diminution in the cytotoxic activity in those animals receiving TCDD treated bone marrow (Kerkvliet et al, 2002).

In 2002, Do et al examined the role that an apoptotic pathway may have in causing thymic atrophy in estrogen treated mice. β-estradiol-17-valerate (E2) was administered to C57Bl/6 wild type, Fas-deficient, and FasL-deficient mice at doses of 0.1 up to 75 mg/kg body weight, or control vehicle. Thymi from these adult mice were harvested on days 1, 4, and 7 after administration. E2 resulted in decreased thymic cellularity in all but the lowest dose (0.1 mg/kg) group. However, the extent of thymic atrophy was less in the Fas-deficient and FasL-deficient mice when compared to the wild type. Analysis for apoptosis demonstrated significantly less apoptosis in the Fas-deficient and FasL-deficient E2 treated mice when compared to the wild type mice, suggesting that the atrophy noted may be caused by an estrogen-induced death receptor pathway and not a mitochondrial pathway. In addition, RTPCR showed an increase in Fas and FasL gene expression in the thymocytes after E2 treatment.

1.9 Effects of TCDD and DES on the developing immune system

The sensitivity of the fetal immune system to toxic exposures that occur during organogenesis or development has been clearly demonstrated to be greater than exposures in the adult (Holladay and Luster, 1996; Holladay, 1999). Included in those agents investigated are TCDD and DES.
Early documentation of the effects of TCDD on gestationally- versus neonatally-exposed F344 rats suggests that the gestational exposure caused suppression of the delayed type hypersensitivity response for longer periods than neonatal exposure in animals having an otherwise normal appearance (Faith and Moore, 1977). Decreases in total body weights, thymus to body weight ratios, and spleen to body weight ratios were noted in treated animals when compared to controls. Statistically significant differences between gestationally and postnatally exposed pups were also noted, with a greater effect seen in those animals exposed during gestation.

To investigate the potential for developmental exposure, Nau and Bass (1981) used radiolabeled TCDD to determine placental transfer in both early and late gestation in NMRI mice. Of the late gestational exposure (gd16), when doses of 5, 12.5, and 25 µg/kg TCDD were administered to the dam orally or by subcutaneous or intraperitoneal injection, fetal tissues contained approximately 1-2% of the level present in the maternal liver. The fetal liver possessed the highest amount measured of the individual fetal tissues assessed and had approximately 2 to 4 times the level of TCDD of the nonhepatic tissues. In early gestation exposures, the highest concentrations of TCDD were noted when the doses were administered on gd 9 or 10. These results demonstrate that TCDD does cross the placenta, albeit in fractions of the maternal exposure. TCDD was able to cross the placenta and was present in milk in concentrations high enough to be measurable in the fetus and neonate. Nau et al (1986) addressed maternal-pup transfer, again using radiolabeled TCDD and exposing some mice gestationally while others received vehicle. By cross-fostering exposed and non-exposed feti with exposed and non-exposed dams, they showed that TCDD is excreted in the milk and results in
exposures of the nursing neonates that are similar to that of the dam. The total body burden of nursing dams decreased as they progressed through lactation.

The distribution of TCDD through embryonic tissues was found to be extremely rapid after oral administration on gestational day 12 of C57Bl/6 mice. Within 30 minutes of oral dosing, TCDD was found in the placenta, embryonic liver, and palate. Blood and placenta levels peaked at 3 hours after administration, while other tissues peaked 8 hours after dosing (Abbott et al, 1996). TCDD levels then decreased slightly in the liver and palate after 8 hours. This rapid distribution and verification of the presence of TCDD after oral gavage ensures that experimental exposures using oral gavage should be reliable in delivery of compound to the fetus rapidly.

TCDD was found to significantly alter the expression of cell surface antigens CD4 and CD8 in gestationally exposed mice (Holladay et al, 1991). The double positive (CD4+CD8+) cells were decreased at very low maternal exposures (1.5 and 3 µg/kg daily from gd 6-14) when evaluated on gd 18. Increases, on a percentage but not numeric basis, in the less mature double negative (DN) cells and the CD8+ single positive cells were also seen. Significant changes in the percentage of CD4+ single positive cells relative to controls were not noted. The increased proportion of DN cells persisted after birth to postnatal day 6.

Another gestational exposure study performed by Blaylock et al (1992) used the same dosing regimen of 0.0, 1.5, or 3.0 µg/kg/day of TCDD for nine consecutive days from gd 6-14, and examination of fetal thymocytes was performed on gd 18. In this study, antibodies to identify the presence of J11d, αβ or γδTcR, and the presence of peanut agglutinin binding (PNA) were used to determine maturation of the thymocytes
after exposure. Both weight and thymus cellularity were decreased by the administration of TCDD. During the maturation from the CD4CD8 double negative stage to the CD4CD8 double positive expression, the TcR switches from the $\gamma\delta$ to the $\alpha\beta$ form. A statistically significant decrease in the more mature $\alpha\beta$TcR and an increase in the less mature $\gamma\delta$TcR was noted in those feti exposed to 3.0 $\mu$g/kg TCDD. A similar difference was noted in the J11d+ cells, a marker which is present on the less mature cortical thymocytes. Complement mediated lysis of the CD4+ cells was used to isolate the CD4CD8 double negative cells and the CD8+ single positive cells. In these remaining cells, the presence of J11d predominated, with over 95% of control, low, and high dose cells identified as J11d+. Likewise, PNA is a maturational marker, and the number of PNA+ cells decreased overall with TCDD exposure. These data support the hypothesis that the decrease in cellularity and alteration in cell surface antigen expression is indicative of a maturation arrest caused by TCDD.

Fetal thymic organ culture was utilized to further identify the targets of TCDD and DES and their effects on the immune system (Lai et al, 1998). The thymus organ cultures were used to separate the direct effects of these immunotoxicants on the thymus from those described for the prolymphoid compartment of the fetal liver. In this experiment, both agents caused a reduction in the double positive cells as well as a lower cell yield over all phenotypes. TCDD also resulted in an increase in the CD8+ single positive cells, though DES led to an enrichment of the double negative cells. In those double negative cell populations, TCDD induced an increase in the more immature stage than DES. TCDD also markedly reduced the expression of RAG-1 and RAG-2, and TdT in CD4CD8$\gamma\delta$TcR- cells compared to controls. DES caused a less dramatic decrease in
only the expression of RAG-1. These results support the notion that DES and TCDD may have distinct mechanisms for inducing thymic atrophy.

DES has been shown to cross the placenta and was found a varying concentrations in fetal tissues (Hill et al, 1980; McLachlan, 1979; Shah and McLachlan, 1976). Transplacental exposure to DES in mice has resulted in diminished reproductive capability of females and increased incidence in cervical, vaginal, or uterine cancer. In the male offspring, genital tract abnormalities were noted at the highest dose administered (McLachlan, 1979). In addition to these reproductive effects, impairment of immune function and immune surveillance have been noted in a variety of prenatally exposed animals (reviewed by Walker, 1989).

1.10 Effects of TCDD and DES related to thymic atrophy

The effects leading to thymic atrophy by these agents are likely multifactorial in nature. Extensive research has been completed on the thymic depletion resulting from TCDD exposure at extremely low doses (µg/kg ranges) (Staples et al, 1998; Silverstone et al, 1992; Kamath et al, 1997; Frazier et al, 1994). DES exerts its effects at similar doses (Holladay et al, 1993). Substantial efforts have been made to identify the potential mechanisms that account for thymic atrophy/hypocellularity. The proposed mechanisms include increased apoptotic events occurring within the intrathymic lymphocyte population, an arrest of maturation of the thymocytes within the thymus with a concomitant inability of thymic lymphocytes to differentiate and expand, and a decreased seeding of the thymus by hematopoietic precursors of either fetal liver or bone marrow.

As the effects of TCDD and DES on the thymus are similar, evaluation to determine if TCDD acted via the ER as well as the AhR was performed. Ovariectomized
or sham-operated BALBc mice were administered 17β-estradiol valerate which results in uterine hypertrophy, thymic atrophy, and a reduction in number of the lymphocyte stem cell markers. These effects were blocked by ICI 164,384, a pure estrogen antagonist. However, treatment with ICI 164,384 did not block the TCDD-elicited thymic atrophy or bone marrow alterations seen in experimental animals. Effects of TCDD are not mediated by estrogen receptors, nor is estrogen required for these effects to occur as ovariectomized mice had the same thymic atrophy and hypocellularity as well as decrease in bone marrow TdT and Rag-1 expression as was found in those animals that were sham operated (Frazier et al, 1994).

Evaluation for apoptosis in the thymic lymphocyte population after dexamethasone (DEX), beta-estradiol (E2), or TCDD treatments has been performed in adult BALBc mice (Silverstone et al, 1994). Dexamethasone is a glucocorticoid with known apoptotic effects in the double positive thymocyte (CD4+8+) subset. Comparisons of the effects of E2 and TCDD with those elicited by DEX on the thymus and bone marrow were made. Apoptotic events were confirmed by the use of thymocyte DNA fragmentation analysis. In this study, DEX induced maximal thymic atrophy earlier (at day 3) with TCDD and E2 having delayed effects. In addition, DNA fragmentation was not noted in either the E2 or TCDD treated animals. These results suggested that apoptosis may not be a substantial cause of the thymic hypocellularity seen after TCDD or estrogen treatment. However, if apoptosis does contribute to the thymic atrophy seen in animals treated by estrogens or TCDD, the phagocytic cells in the thymus may be able to remove apoptotic cells and debris at a rate which impedes their detection. Other studies using TCDD (Kamath et al, 1997) suggest early apoptosis (within 8-12 hours of
treatment). However, no significant apoptosis could be detected at 24 hours or later to the end of the study (at 120 hours). These results support thymocyte apoptosis as one of the mechanisms responsible for the decrease in thymocyte or prolymphoid populations seen with these two agents.

However, not all research supports thymocyte apoptosis as the primary cause for thymic atrophy. Inhibition of thymocyte proliferation in the thymic cortex was noted after DES administration, however this effect was not linked to a down regulation of cyclin D3 (Gould et al, 2000). The use of various rat strains in this study also demonstrated that genetic variation in the subjects resulted in differences in DES sensitivity. These findings indicate the importance genetic factors in modulating DES's effects in the thymus.

Transgenic mice have afforded researchers the opportunity to investigate the molecular effects of TCDD, estradiol, and dexamethasone with a target of overexpression of a single gene. Dexamethasone (DEX), estradiol, or TCDD were administered by injection to lck$^{\text{pr}}$-Bcl-2 and wild type mice, and the mice were sacrificed at various times after exposure using age matched controls for comparison. The Blc-2 transgenic mice have been shown to be resistant to the effects of DEX-induced thymic atrophy as Bcl-2 is expressed throughout development, instead of primarily during the double-negative and late single-positive stages in wild type mice. Thymic atrophy and recovery were measured for up to one month post exposure. In addition, the TUNEL assay was used to detect apoptosis in the thymocytes. Results of this experiment confirmed that Bcl-2 transgenic mice were protected after a single dose of DEX. Atrophy was still induced with estradiol and TCDD, suggesting a different mechanism of thymic atrophy for these
two latter immunotoxicants. Both TCDD and estradiol caused shifts in the CD4CD8 subsets in the transgenic mice which were similar to those seen in wild type mice with the same treatment. However, in the TUNEL assay, no signs of apoptosis were detected at 0.5, 1, 2, 5, 6, or 9 days after TCDD or estradiol treatment, even at higher doses. When recovery kinetics were compared, estradiol-treated animals were comparable to controls in cellularity by the end of a month, while TCDD treated mice continued to have marked thymic atrophy. Notably, the transgenic mice took longer to recover than the wild type mice did after similar treatment. In addition to differing recovery kinetics, DEX caused a marked decrease in the CD4CD8 double positive cells by 96% over controls, while the estradiol and TCDD treated mice showed a 27% and 10% decrease in the double positive thymocyte population respectively. Dexamethasone-treated mice also showed substantial increases in the percentages of CD4 and CD8 single positive cells, while the increases in CD4 single positive cells were slight in estradiol and TCDD treated mice. The CD8 single positive cells were affected by estradiol and TCDD. However the increases were not to the same degree as those in the DEX-treated thymuses (Staples et al, 1998). The results of this experiment suggested that the mechanism for dexamethasone-, TCDD-, and estradiol-induced thymic atrophy are distinctly different.

Use of Bcl-2 transgenic mouse fetal thymic organ culture to examine the effects of immunotoxicants on the developing organ in isolation was performed (Lai et al, 2000). Thymocyte development was assessed after exposure to TCDD and DES in both Bcl-2− and Bcl-2+ cultures. The Bcl-2+ cultures showed no protection against the decreased cellularity produced by TCDD and the decrease in cellularity of both the transgenic positive and negative cultures were similar. DES also caused a decrease in thymic
cellularity, although the Bcl-2+ culture was not as dramatically affected suggesting that Bcl-2 may mitigate the inhibition of thymic development induced by DES, but not by TCDD. TCDD significantly decreased the early developmental prothymocytes populations, c-kit CD44+CD25+ and c-kit CD44 CD25+, while DES increased the c-kit CD44 CD25+ subset of cells. The point in the cell cycle at which DES and TCDD seemed to have an effect was determined, with TCDD causing a decrease in S-phase cells. DES caused an increase in G2/M-phase cell in treated FTOC which was similar in both Bcl-2+ and Bcl-2- thymocytes. In this experiment, DES also induced apoptosis in Bcl-2- FTOC, but not in Bcl-2+ FTOC. This apoptosis was seen primarily in the double negative immature cell population. However, no detectable apoptosis was noted after TCDD exposure from 12 to 120 hours after exposure in either the Bcl-2- or Bcl-2+ cell cultures.

An in vitro study of fetal thymus organ culture of gd 15 Balb/c mice maintained in either control medium or DES showed a marked diminution in total thymocyte number of those exposed to DES. While total losses in each of the phenotypes identified by CD4 and CD8 were noted, increased losses were seen in the CD4+CD8-, CD4+CD8+, and CD4+CD8+ subsets. Developmental stages identified showed that nearly all of the CD4- CD8- CD3- triple negative thymocytes were of the earliest population. This study supports an arrest in maturation as a possible mechanism for DES effects on thymic atrophy (Rijhsinghani, et al, 1997)

1.11 Molecular targets of TCDD and DES

Because many of the effects of DES and TCDD are similar, research into the basis of their immunotoxicity has often been simultaneously performed. Both of these
agents form receptor-ligand complexes which then translocate to the nucleus to affect estrogen responsive or xenobiotic responsive elements associated with gene transcription. However, while similarities in their immune alterations have been documented, significant differences have also been noted. This suggests that in addition to acting via separate and distinct receptors, these two agents may affect the cell via different mechanisms as well.

The above collective reports support the possible dysregulation of gene expression by TCDD or DES at critical points in the cell cycle. Either an increase in pro-apoptotic events, a decrease in anti-apoptotic gene expression, or an alteration in ratios of genes controlling cell death may be responsible for this proposed mechanism of decreased thymic cellularity. In addition, genes regulating the cell cycle in rapidly proliferating tissues as seen in the fetus may be expressed in increased or decreased levels after TCDD or DES exposure leading to potential maturation arrest and impedance of further replication.

The genes of interest in this study are Bcl-2, p53, PKCα, and c-jun. These were chosen because of their well documented and prominent roles in regulation of the cell cycle and apoptosis. Gene p53 is described as a growth suppressor or pro-apoptotic gene in its activity (Elledge and Lee, 1995). The functional mechanism blocks progress from G1 to the S phase of the cell cycle, preventing proliferation. Bcl-2 has been characterized in the mouse and is expressed in all tissues of the neonate, but found at high levels in the thymus and spleen only in adult mice (Negrini et al, 1987). The function of Bcl-2 appears to involve the mediation of apoptotic events and promotion of cellular proliferation. Bcl-2 is expressed in high amounts in the surviving medullary thymocytes
of the thymus, while it is nearly absent in the cortical thymocytes that typically undergo normal physiologic apoptosis. The hematopoietic cells that descend from the stem cells express Bcl-2, however this gene product is often absent in the terminally differentiated cells. The Bcl-2 gene is also present in epithelial tissues which undergo hyperplasia or involution, supporting its role in proliferative or apoptotic responses (Korsmeyer, 1992).

PKCα does not directly inhibit or promote apoptosis, but it is responsible for the phosphorylation of specific amino acid groups on other signaling molecules. Many of the cell cycle promoters/inhibitors are either activated or inactivated by phosphorylation. By either increasing or decreasing the levels of PKCα, phosphorylation, and therefore activation status, may be affected in many of the cell signal pathways. In this way, an elevation or diminution of PKCα expression may alter the progression of a cell through the cell cycle, affecting its ability to proliferate or differentiate (Boulikas, 1995). A similar function in mediation of cellular responses to cytokines and growth factors has been described for c-jun (Barr and Bogoyevitch, 2001).

In addition to gene expression studies, microscopic evaluation of the fetal thymus and liver was performed as part of the present work. TCDD and DES clearly target these tissues, however histologic changes of these tissues after exposure remain poorly described. Decreases in total cellularity and alterations in cellular composition have been reported. However, comparisons between these changes and the light microscopic presentation of these tissues after treatment have not been published. These histologic studies may support observations made as part of the proposed gene expression studies.

Flow cytometry of the thymocytes was also used to further characterize the changes of the lymphoid population of the thymus, and comparisons between treatment
groups and control animals will be made. Markers used characterized thymocyte maturation [i.e. transitions from immature double negative (CD4 CD8−), to double positive (CD4+CD8+), and single positive thymocytes (CD4+ or CD8+)]. The apoptotic marker 7-aminoactinomycin D (7-AAD) was used as a third simultaneous stain, to detect potential chemical effects on apoptosis of thymocytes across all phenotypes defined by the CD4 and CD8 surface antigens. Alterations noted were correlated with the outcomes of histology experiments.

Table 1.1 Summary Table
Comparison of Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>TCDD</th>
<th>DES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induce immune dysregulation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Induce thymic atrophy</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Decrease thymocyte cellularity</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Induce thymocyte apoptosis</td>
<td>+ / -</td>
<td>+ / -</td>
</tr>
<tr>
<td>Alter CD4CD8 subpopulations (increase DN, decrease DP)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Decrease T-cell progenitors (in hematopoietic compartment)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alter fetal thymus architecture</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Induce hepatic enzymes/hyperplasia (in adults)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alter fetal liver morphology</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alter fetal liver gene expression</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

"+" historic data demonstrate this effect
"-" historic data do not demonstrate this effect
"+ / -" historic data are inconclusive/contradictory
ND Not done in historic studies
1.12 Hypothesis

Both TCDD and DES effect many of the same alterations in the fetal mouse thymus and T-cell progenitors. After gestational exposure, fetal thymocyte hypocellularity is noted with decreases in thymic weights, as well as alterations in the thymocyte subpopulations as identified by CD4 and CD8 cell surface antigens. While increases in the percentage of CD4CD8 DN cells are noted within the thymus and concomitant decreases in the more mature CD4CD8 DP thymocytes, decreases in numbers of T-cell progenitors are found in the fetal liver or adult bone marrow after treatment with either compound compared with controls.

Historically, histologic evaluation of fetal thymus has not been described, although alterations in organ architecture have been described in adult mice after treatment with either TCDD or DES. Additionally, hepatic enzyme induction, reparative hepatocellular hyperplasia, and other morphologic changes in the liver are noted in adult animals, however these have not been described in fetal mice.

We hypothesize that these two compound, while acting via separate ligand-receptor complexes, target similar cellular pathways and share gene targets. In addition, we hypothesize that histologic alterations, similar to those seen in adult mouse thymus and liver, occur in the fetus after exposure to either TCDD or DES during late gestation. To test this hypothesis, we exposed gd 14 and 16 C57Bl/6 mice to 5 or 10 µg/kg TCDD, 48 µg/kg DES or corn oil vehicle by oral gavage. Tissues were harvested on gd 18 and experiments performed on these tissues.
References


Kerkvliet NI, Shepherd DM, Baecher-Steppan L, 2002. T-lymphocytes are direct aryl hydrocarbon (AhR) targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): AhR expression in both CD4+ and CD8+ cells is necessary for full suppression of a cytotoxic T lymphocyte response by TCDD. Toxicol Appl Pharmacol 185:146-152.


