Immunomodulatory Effects of Diethylstilbestrol During Prenatal and Adult Life

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(ABSTRACT)

For nearly forty years diethylstilbestrol (DES) was administered to pregnant women to maintain healthy pregnancies. During this time, it is estimated that several million men and women have been exposed to DES during sometime of their life. The most common period of exposure was during fetal development. Although rarely used for the maintenance of pregnancy now, its current medical use is restricted to certain clinical situations such as breast and prostate cancer therapies in adults. Thus, DES exposure spans the entire lifetime, from prenatal to geriatric age. Since the early 1950s, health risks were beginning to be associated with prenatal DES treatment. So far only reproductive problems such as infertility, neoplastic diseases of the cervix and vagina and testicular cancers have been well-documented in DES cases. Immunological abnormalities associated with DES are only now beginning to be recognized. Self-reported cases and questionnaire-based studies have revealed increased incidence of infections and autoimmune diseases in DES exposed people. Animal studies that have examined the immunological effects of DES treatment are largely restricted to one gender, or to one dose of DES or to the developmental period. This is an important issue since human exposure to DES occurred in both men and women, at all ages and, at a wide-range of doses. The purpose of these studies was to investigate the immunological consequences resulting from the exposure to DES. Since sensitivity can vary between genders, dose and at the time of exposure, it is critical to investigate the DES-induced immunological changes during all stages of life in both genders. To address these critical gaps in the literature, we examined the immunomodulatory effects of adult and prenatal exposure to DES in males and females. Our findings show that DES effects were evident in both the thymus and spleen. DES markedly affected the apoptosis of thymocytes and the ability of splenic lymphocytes to proliferate in response to stimulants and secrete vital cytokines such as interferon-gamma. Our notable findings were that *in-utero* exposure to DES resulted in profound alterations in lymphocyte functionality, which were noticed as late as one-year of age. This suggests that alterations to the *in utero* environment can have deleterious consequences that may be long lasting. These studies have profound implications to the humans and animals exposed to DES, and indirectly to a whole range of other estrogenic compounds.

Dedication

I would like to dedicate my Master's thesis to my Husband, Martijn, my Family,
Mom, Dad and Jody, and to my Friend, Andrea.
We did it sweetie! Ik hou van jou.

Acknowledgements

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List of Abbreviations

7-AAD	7-aminoactinocycin D
BSA	bovine serum albumin
CD	cluster of differentiation
Con A	conconavalin A
DES	diethylstilbestrol
DEX	dexamethasone
ELISA	enzyme linked immunosorbent assay
ER	estrogen receptor
FITC	fluorescein isothiocyanate
IFNγ	interferon gamma
LPS	lipopolysaccharide
PE	phycoerythrin
PMA+I	phorbol myristate acetate + ionomycin
SLE	systemic lupus erythematosus

Introduction and Rationale

Exposure to estrogenic compounds can occur routinely. These compounds, also known as estrogenic endocrine disruptors, exist naturally as synthetic drugs (diethystilbestrol), phytoestrogens (soybean, isoflavones), pesticides (methoxychlor), industrial chemicals (octylphenol, nonylphenol), consumer products (cosmetic creams), culture media reagents (phenol red) and endogenous hormones. Estrogenic compounds have also been produced synthetically for pharmaceutical uses. Endocrine therapy has been used for several decades for various health care purposes. Recent clinical trials have prescribed hormones (progesterone) to pregnant women who are at-risk of premature delivery. Other uses for therapeutic hormones include many reproductive maladies such as menopause related discomforts, breast cancer and prostate cancer. Those who are exposed to estrogenic compounds span the entire population including females and males exposed from prenatal development (mothers received hormones during pregnancy) to older populations (who are at higher risk of developing breast or prostate cancers). Although, endocrine therapy has been in practice for many years, no one has thoroughly investigated the effect of exogenous hormones on overall health. These studies explore the immunological consequences of exposure to a widely prescribed estrogenic compound, diethylstilbestrol. By 1992, over 5 million people were reported to have been exposed to DES some time during their life. Therefore, it is important to investigate the cellular immune response to DES treatment during all stages of life from *in-utero* development to adult age. Thus far, the majority of immunological related DES studies in animals are restricted to examining the DES induced immunological effects in one gender, exposed to one dose and mostly during neonate life. *We reason that since the immune competence varies with age, the sensitivity of the immune system to DES may differ relative to age.* These studies will address the above gaps in the literature by examining the immunological DES induced effects in mice exposed to several different doses during adult and prenatal stages of life in both females and males.

Chapter 1: Literature Review

<u>Diethylstilbestrol and Historical Perspectives</u>

Diethylstilbestrol (DES) was synthetically developed shortly after the biological understanding of the role of estrogenic compounds in 1938. The chemical manufacturing of DES was based on the structure of natural estrogen, 17-β estradiol (**Figure 1.1**). The estrogenic potency of DES was determined to be five times that of natural estrogen

Figure 1.1 Chemical structures of natural estrogen, 17- β estradiol, and synthetic estrogen, diethylstilbestrol
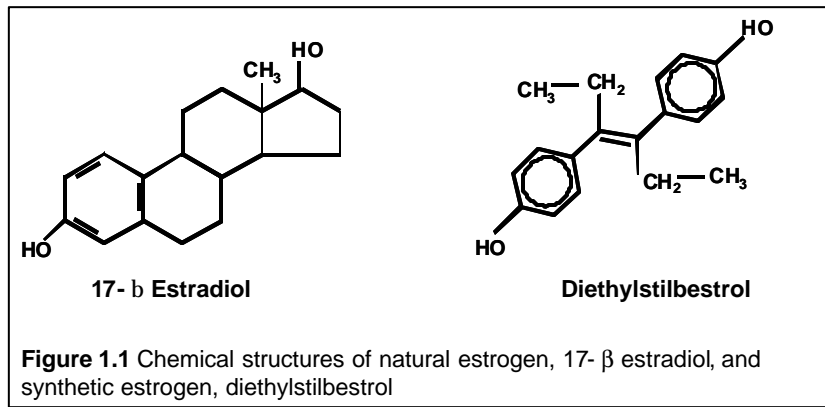
(Marselos and Tomatis, 1992). It was therefore believed that the increased estrogenic potency of DES would replace and/or better regulate the natural level of estrogen in pregnant women who were at-risk of developing serious pregnancy-related complications and threatened spontaneous abortions. In just two years after its production, DES was expediently introduced into clinical medicine under various trade names (**Table 1.1**). DES was mistakenly or

Table 1.1 Trade names used to prescribe DES
DES Trade Names
desPLEX
Dibestil
Fosfestrol
Stilboestrol
Stilphostrol
DES was prescribed to pregnant women from 1940-1971 under a variety of trade names. Table 1 is short list of the common DES trade names used.

perhaps callously advertised as a drug that assists in having healthy babies. Based on clinical records, it is estimated that 2-4 million women were

exposed to DES during pregnancy and millions more were exposed *in-utero* during 1940-1971. Since DES was approved for therapeutic use in humans soon after its production, a thorough investigation of its dose efficacy and its clinical side effects was never adequately conducted. Consequently, a wide range of dose (1.4g –17.9g) was administered to women during all stages of gestation (Cunha et al., 1999). Despite a landmark study published in the early 1950s, which proved DES was ineffective of reducing the occurrence of spontaneous abortions, DES was still prescribed to women until 1983 (Dieckmann et al., 1953). In addition to its clinical uses, DES was also given to nearly 80% of U.S. cattle and sheep as a growth promoter until 1979 (Marselos and Tomatis, 1992). Hence, occupational exposure to DES is likely to have occurred in cattle and sheep farmers.

Diethylstilbestrol Effects on the Reproductive Tissues

Retrospective studies based on patient medical records have consistently shown adverse reproductive effects due to DES exposure, particularly when exposure occurred during prenatal life. Prenatal DES exposed women are at high risk of developing a variety of reproductive disorders including non-neoplastic (infertility, vaginal hyperplasia), pre-cancerous (vaginal epithelial cell dysplasia) and neoplastic (adenocarcinomas of the cervix and vagina) diseases (Stillman, 1982, Sandberg et al., 1981, Burke et al., 1981). Similarly, men prenatally exposed to DES have been noticed to be at risk of low sperm count and possible development of testicular cancers (Golden et al., 1998). DES is one of the few chemically synthesized compounds that are now regarded as a transplacental carcinogen and a teratogen. Prenatal and neonatal exposure of laboratory rodents, monkeys and dogs to DES has confirmed the carcinogenic and teratogenic potential of DES in reproductive organs (Newbold and McLachlan, 1982, Walker, 1989, Fielden et al., 2002). DES has been shown to induce reproductive abnormalities through the binding of estrogens receptors (Korach and McLachlan, 1985). DES has a high affinity to estrogen receptors making it possible to target reproductive tissues. The availability of estrogen receptor knockout mice has made it possible to study the mechanisms of DES

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induced effects. Studies have shown that neonatal DES exposed alphaERKO mice did not present the characteristic reproductive abnormalities that are commonly observed in DES treated mice suggesting that the alpha subset of the estrogen receptor is vital for DES induced effects (Couse et al., 2001, Prins et al., 2001). Since estrogen receptors are also present on immune cells, it is important to investigate the immunological effects of DES treatment in ERKO mice as well. This aspect has yet to be explored.

Diethylstilbestrol Effects on the Immune System

　　a.) Clincal Observations:

In addition to the many DES-associated reproductive abnormalities, immune consequences of DES exposure to humans are also now apparent. These findings are not surprising, considering that 17-β estradiol has profound effects on the immune system (Ahmed, 1994, Ahmed et al., 1999). Women prenatally exposed to DES who presented evidence of vaginal epithelial changes tend to develop a variety of autoimmune diseases (Noller et al., 1988). A questionnaire-based study involving DES exposed mothers, daughters and sons revealed that respiratory infections (i.e. flu and cold), asthma, arthritis and lupus were reported more frequently in DES exposed people (Wingard and Turiel, 1988). In 1998, another study was published which showed that prenatally exposed women were more susceptible to measles and bladder infections suggesting DES impaired the immune response (Vingerhoets et al., 1998). *In-vitro* studies on peripheral blood mononuclear lymphocytes of DES daughters have shown altered mitogen-induced responses including decreased natural killer cell activity, and increased proliferative response to T cell mitogens (Con-A, PHA) and IL-2 (Ways et al., 1987, Burke et al., 2001).

b.) Animal Studies:

Similar DES-induced immune alterations have also been noted in animal studies. DES treatment in mice has led to altered proliferative response to T-cell mitogens (Kalland et al., 1979), decreased natural killer cell functions (Kalland, 1984), impaired immunity against infections (Kittas and Henry, 1980, Luebke et

al., 1984, Fugmann et al., 1983), and spontaneous autoantibodies to a negatively charged phospholipid, cardiolipin in outbred NMRI mice (Forsberg, 2000).

DES may also be responsible for stem cell alterations since murine studies have shown that DES associated ailments were also present in the F_2 generation (Walker and Kurth, 1995, Walker and Haven, 1997). For example, the incidence of DES induced vaginal adenocarcinomas in the F_2 generation was comparable to the F_1 generation. This implies that the direct exposure to DES is not necessary to induce reproductive neoplasias. These transgenerational effects of DES raise concern by suggesting that DES-altered genes can be inherited. Taken together, these studies clearly show that although DES has been banned for nearly twenty years, its adverse effects may continue to be present in surviving generations. Further studies on DES exposure are important considering that DES effects on the immune system are not intensively investigated. It is likely that immune related abnormalities may become evident in the future.

How must DES impair the immune system in order to induce the variety of immunological problems that have been associated with DES treatment? One possibility is that DES could directly alter the development of T cells. For instance, a number of animal studies have shown that DES has adverse effects on the thymus, a T cell developmental organ. Primarily, the DES induced pathological effect includes atrophy of the thymus and marked decrease in the number of thymocytes (Forsberg, 1984, Forsberg, 1996, Hirahara et al., 1994,
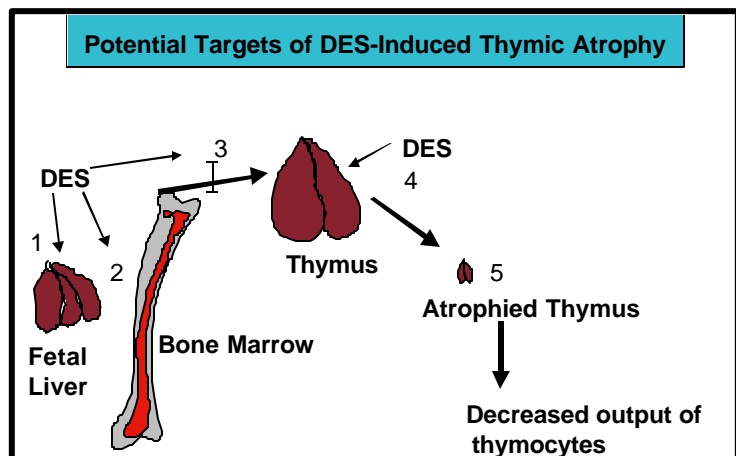
Figure 1.2: DES may induce thymic atrophy at many levels. DES may act on the fetal liver (1) or bone marrow (2) of adults to alter the hematopoetic microenvironment and affect at the downstream developmental events of stem cells that are destined to become prothymocytes. DES could affect the migration of prothymocytes to the thymus (3). DES could act on the thymus directly to affect the thymic development (4). It is possible that DES may act on the thymic stromal cells to affect the critical development of thymocytes. Direct effect of DES on the thymocytes to induce apoptosis is possible, particularly on the highly immature population. The overall result of DES effects on the thymus results in decreased output of thymocytes (5).

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Holladay et al., 1993, Rijhsinghani et al., 1997). The mechanisms underlying DES induced atrophy of the thymus are a subject of intense investigations. It is possible that DES could affect the thymus at many levels (**Figure 1.2**). DES may act on the fetal liver and decrease the output of pro-thymocytes specifically homing to the thymus (Holladay et al., 1993). In this study, prenatally DES exposed mice had significant thymic atrophy yet showed no signs of impaired thymocyte viability or ability to mature to CD4$^+$ or CD8$^+$ cells. Since there was no apparent direct effect of DES on the thymocytes, fetal liver hematopoetic cells were analyzed for depletion of pro-T and pro-B cells, which could possibly lead to the observed DES induced thymic atrophy. This study reported that prenatal exposure to DES depleted Mac1$^+$ and TdT$^+$ lymphocytes, yet had no effect of CD45R$^+$ (B220) lymphocytes in the fetal liver suggesting that DES targeted cells of macrophage lineage and pro-thymocytes. In an adult animal, DES could affect the pro-thymocyte emigration by acting on the bone marrow (**Figure 1.2**). Another way DES could induce thymic atrophy is by acting directly on the thymus to influence the thymic architecture or the microenvironment. It is conceivable that DES could act on the thymic epithelial cells, which possess estrogen receptors including alpha estrogen receptor to which DES has been shown to have affinity. Although it may seem unlikely, based on the available literature, DES could induce thymic atrophy through direct induction of thymocyte apoptosis. For instance, a study from our lab showed direct exposure of thymocytes to DES did not induce apoptosis of thymocytes as determined by six different assays for apoptosis (Donner et al., 1999). However, there is evidence that DES can induce thymocyte apoptosis in immature populations. Culturing of fetal thymic organ cultures in the presence of DES also revealed that DES decreased CD4$^-$CD8$^-$ immature thymocytes, suggesting that DES targets the immature thymocyte population (Lai et al., 2000). In addition, a short-term exposure to DES in young adult mice caused apoptosis of CD4$^+$CD8$^+$ immature thymocytes (Calemine et al., 2002).

DES may also affect mature T cell functions. The possibility that DES could affect the immune repertoire by altering the secretion of cytokines is an attractive

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hypothesis that is based on observations noted in 17-β estradiol treated mice. Studies from our laboratory as well as others (Sarvetnick and Fox, 1990) have shown that 17-β estradiol up regulate interferon-gamma (IFNγ) mRNA gene expression and secretion of IFNγ protein (Karpuzoglu-Sahin et al., 2001b). Therefore, it is conceivable that DES can also alter IFNγ, a prototype Th-1 cytokine. Alterations of IFNγ are significant, since the cytokine is considered to be a master cytokine that affects the functioning of all the key cells of the immune system and affects the course of several autoimmune diseases (Haas et al., 1998, Hayashi et al., 2001). Studies from our laboratory have shown that prenatally DES exposed mice, given a second exposure to DES as late as 1 to 1.5 years of age, revealed that the immune system of these mice responds abnormally to T cell stimulants. Splenic lymphocytes from these mice when exposed to T cells stimulants (Concanavalin-A (ConA) or anti-CD3 antibodies and anti-CD28 antibodies) secreted abnormally high levels of IFNγ (Karpuzoglu-Sahin et al, 2001a). These results suggest that exposure to DES during the critical fetal developmental period permanently alters the immune system of these mice to respond to T cell stimulants. Together, these studies show that DES, in addition to being a teratogen, carcinogen and a reproductive hormone, is also an immune altering drug. Therefore, it is conceivable that prenatal DES treatment could be linked to a variety of immunological abnormalities. DES induced dysfunctional T cell development is likely to affect mature T cell competence and contribute to immune related illnesses.

Today, DES is still used as an experimental drug for prostate and breast cancer therapy as well as for urinary incontinence in veterinary clinics, despite the many associated adverse immunological and reproductive effects reported in DES exposed people. By 1992, over 5 million people were reported to have been exposed to DES some time during their life. Therefore, it is important to investigate the cellular immune response to DES treatment during all stages of life from *in-utero* development to adult age. Thus far, nearly all immunological related DES animal studies are restricted to examining the DES induced immunological effects in one gender, exposed to one dose and mostly during

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neonate life. We reason that since the immune competence varies with age, the sensitivity of the immune system to DES may differ relative to age. These studies will address the above gaps in the literature by examining the immunological DES induced effects in mice exposed to several different doses during adult and prenatal stages of life in both females and males.

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Chapter 2

Immunomodulation by diethylstilbestrol is dose and gender related: Effects on thymocyte apoptosis and mitogen-induced proliferation

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Key words: Diethylstilbestrol (DES), immunity, gender, dose, proliferation, and apoptosis

ABSTRACT

It is believed, but not proven, that the immunomodulatory effects of DES may vary with the dose and/or gender. To address these critical gaps in the literature, DES was administered to female and male CD-1 mice as four subcutaneous injections for one week at 0, 5, 15, and 30μg/kg bw doses, and immunological and reproductive effects examined a day after the last injection. Female thymuses were significantly larger than their male counterparts. Short-term administration of DES to female or male mice neither induced thymic atrophy nor altered the relative percentages of thymic subsets. Nevertheless, DES treatment of female or male mice induced a dose-related apoptosis of $CD4^+8^+$, $CD4^+8^-$ and $CD4^-8^+$ subsets as analyzed by 7-amino-actinomycin D (7-AAD). Immature $CD4^-8^-$ subset of thymocytes from females was also affected by high dose DES. The pattern of mitogen-induced proliferation of splenic lymphocytes varied with the dose of hormone and the gender. In females, splenic lymphocytes from low dose DES (5 μg/kg bw)-treated mice exhibited an increased proliferative response to Con-A, LPS or PMA/ionomycin compared to controls. Similar cultures from mice treated with higher doses of DES (15 or 30 μg/kg bw) did not manifest an increased proliferative response, but rather showed a trend for suppressed proliferation, especially in response to Con-A. In males, DES had minimal effects with the exception of increased proliferative response to Con-A in splenocytes from medium-dose-DES-treated mice. The changes in mitogen-induced proliferation in DES-treated female mice were not mirrored by similar changes in the relative numbers of $CD90^+$ or $CD45R^+$ cells, or in ratios of anti-apoptotic Bcl-2 to apoptotic Bax proteins. Con-A-activated splenocytes from DES-treated mice, particularly from females, had a decreased ability to secrete interferon-γ compared to controls. Taken together, these findings suggest that short-term

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exposure to DES has differential immunological effects depending upon the dose of hormone and sex.

INTRODUCTION

A synthetic form of estrogen, diethylstilbestrol (DES), is believed to have been prescribed to 2 to 4 million women with high-risk pregnancy during 1940 - 1971. Other potential sources of human exposure to DES include occupational exposure of cattle and sheep farmers (who used DES to promote growth of these animals) and possibly consumers of DES-contaminated meat. It is believed that nearly 80% of all cattle and sheep in the USA were given DES as a feed supplement or as an implant during the 1950s through late 1970s (Marselos and Tomatis, 1992).   Unfortunately, it is difficult to precisely estimate how many people were exposed to DES through this source.

Adverse consequences of DES treatment in women became apparent in the late 1960s. DES was the first prescribed drug that was subsequently recognized as a transplacental teratogen. Women who were prenatally exposed to DES were vulnerable to a variety of maladies. These include increased risk of developing a rare neoplasm, clear cell adenocarcinoma (Herbst et al., 1971), and other structural abnormalities in reproductive tissues. Prenatally DES exposed women also have an increased tendency for spontaneous abortion, ectopic pregnancy, premature deliveries, and stillbirths (Marselos and Tomatis, 1992). It is estimated that pregnant women received as little as 1.4 g to as high as 17.9 g of DES (ConA et al., 1999). Adverse reproductive effects of DES tend to vary with the dose administered (Cunha et al., 1999). DES-induced reproductive abnormalities have been replicated in animal studies. For example, studies in

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outbred CD-1 mice confirmed that DES induces vaginal adenocarcinoma as well as other reproductive abnormalities (Newbold and McLachlan, 1982; Newbold et al., 1990; Newbold et al., 1994; Newbold et al., 1997; Newbold et al., 1998; Newbold et al., 2001). Minimal data are available for men who were prenatally exposed to DES. However, it appears that men prenatally exposed to DES may also be at risk, since abnormalities in their reproductive tracts, low sperm count and testicular cancers have been reported (Golden et al., 1998; Marselos and Tomatis, 1992; Degen and Bolt, 2000). Due to the adverse consequences of DES exposure, this estrogen was prohibited for clinical use in pregnant women in 1971 in the USA and was also banned in beef cattle in 1979 (Marselos and Tomatis, 1992). The current therapeutic use of DES is restricted to the treatment of prostate cancer, breast cancer, and in veterinary clinics for urinary incontinence. Despite the banning of DES use in pregnant women, studies addressing the consequences of DES exposure continue to be of topical concern since adverse effects of DES may be long lasting and/or vertically transmitted. For instance, murine studies have shown that DES-associated ailments were also present in the F_2 generation, thereby suggesting an inheritance of DES-altered genes (Walker and Kurth, 1995; Walker and Haven, 1997). Further, illegal use of DES continues to be of concern worldwide. Recently, Swiss authorities have found imported shipments of American beef contaminated with DES (Ingersoll, 2000).

It is now apparent that DES has been associated not only with neoplastic and reproductive disorders, but also with immune abnormalities in humans. Women prenatally exposed to DES appear to be more susceptible to develop a variety of autoimmune diseases (Noller et al., 1988). However, a smaller preliminary report in DES-exposed (n=253 sons and 296 daughters) individuals based on self-reported cases of autoimmune diseases did not find an association of these disorders with prenatal DES exposure (Baird et al., 1996). The authors

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acknowledge that larger sample sizes are needed to definitively link prenatal DES exposure to autoimmune diseases, since these disorders are relatively rare. Also, this study was based on self-reported cases, which inadvertently introduce bias. An epidemiological questionnaire-based study involving 1,700 DES-exposed respondents (including mothers, daughters and sons) revealed that respiratory infections (flu, cold) asthma, arthritis, and lupus were reported more frequently in DES exposed people (Wingard and Turiel, 1988). DES-exposed sons in particular have a higher incidence of immune problems. In a more recent questionnaire-based study, however, DES-exposed daughters (n=170) reported a significant susceptibility to bladder and measles infections compared to control subjects (n=123), suggesting impaired immune responses (Vingerhoets et al., 1998). Other studies have also shown that peripheral blood mononuclear cells from prenatally women exposed to DES had decreased natural killer cell activity (Ways et al., 1987; Ablin et al., 1988).

Animal studies especially in CD-1 and NMRI mice have confirmed that DES alters the immune system. Prenatal DES-exposed mice had diminished prothymocyte stem cells (Holladay et al., 1993) and transiently suppressed antibody responses especially in the female offspring (Luster et al., 1978). DES treatment of neonatal or geriatric mice resulted in diminished antigen-specific delayed type hypersensitivity response (Kato et al., 1988; Forsberg, 2000), reduced proliferative response to T-cell mitogens (Smith and Holladay, 1997; Kalland et al., 1979), decreased graft versus host reaction, or decreased cytotoxic response to mammary tumor virus and natural killer cell function (Kalland, 1984). DES-treated mice had impaired immunity against a variety of infections (Kittas and Henry, 1980; Luebke et al., 1984) ; Fugmann et al., 1983). Other studies have shown that DES also dysregulated the immune system. For example, neonatal DES treatment of male NMRI strain of mice induced autoantibodies to cardiolipin

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(Forsberg, 2000). DES-treated mice had initial hyperstimulation of spleen immune response by 1 month of age, followed by a decline in immune response by 2 to 9 months of age (Ways et al., 1980). The above studies were largely restricted to one gender, or to one dose of DES or to the developmental period. This is an important issue since clinical human studies suggested that DES effects appeared to vary with the dose administered. Thus far, there are no studies that comprehensively address the immunological effects of DES in reproductively mature young animals, relative to dose and/or gender. This study therefore addressed these issues by using an outbred strain of mice, CD-1, which has been shown to be a relevant murine model for human disease (Padilla-Banks et al., 2001).

MATERIALS AND METHODS

Mice: Seven to eight-week-old male and female CD-1 outbred mice (Charles River Laboratories, Wilmington, MA) were housed in groups of four and fed a commercial pelleted diet devoid of synthetic estrogens (Special diet #7013 Harlan Teklad, Madison, WI), and given water *ad libitum.* The animals were housed at a 14 / 10 hour light-dark cycle and subsequently terminated via cervical dislocation. All procedures were in accordance with the guidelines of the animal care committee of the Virginia Polytechnic Institute and State University.

DES injections and Experimental Design: DES was dissolved in autoclaved Tocopherol stripped-corn oil (ICN, Auro, Ohio). The Tocopherol-stripped corn oil chemical composition was as follows: % by weight- Glycerides-98.8%, unsaponifiable matter-1.2%, free fatty acids- 0.07%. It contained only trace amounts of Squalene and carotenoids, 0.02% of ubiquinone, stigmasterol-

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0.07% and campestrol-0.2%. Des in this autoclaved corn oil was administered subcutaneously into the subscapular region. Mice were given 4 injections of 5, 15, 30 μg/kg body weight of DES in 50 μl on alternate days and referred to as low, medium, and high dose, respectively. Mice that received oil vehicle alone served as controls. The experiment was controlled for error by using the generalized randomized complete block design (Lenter et al., 1993).

Effects of DES on reproductive and lymphoid organ weights: Reproductive organs (uterus from females, seminal vesicles and testes from males) and lymphoid organs (thymus and spleen) were isolated, trimmed of all excess body fat and blotted dry of any excess blood using sterile gauzes. The tissues were aseptically transferred to sterile preweighed petri dishes and weighed using an Ohaus-Precision Standard balance (Florham Park, NJ). To assess the morphological effects of DES on the reproductive system, the uteri and seminal vesicles, were preserved in formalin and subjected to histopathological processing. The tissues were routinely processed for paraffin sections, sectioned at 6 microns, and stained with hematoxylin and eosin. From each slide, six glandular lumina were evaluated for height of epithelium. These were selected from glands with round cross sections to avoid the problems associated with the tangential cut sections. The height of the uterine epithelial cells was measured by the micrometer. The slides were read in a coded fashion and decoded later for statistical analysis.

Isolation of lymphocytes: Lymphocytes from thymuses and spleens were aseptically dissociated by gently grinding these organs on a sterile steel wire mesh (Sigma) in phenol red-depleted RPMI-1640 incomplete media (Mediatech, Herndon, VA) by procedures reported previously (Ansar Ahmed et al., 1994; Donner et al., 1999; Verthelyi and Ansar Ahmed, 1998).

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Splenocytes were treated with Tris-ammonium chloride-lysis buffer (pH 7.2) at room temperature to remove erythrocytes before culturing. The cell suspensions were washed twice at 200 Xg for 5-8 min at 4^0C and resuspended in 10 mL of complete media. The complete media consisted of 10% heat-inactivated fetal bovine serum that was pretreated with charcoal and dextran (Atlanta Biologicals, Atlanta, GA) to eliminate any estrogenic compounds in the serum. The media also contained 200 mM L-glutamine, 5000 IU/mL penicillin, 5000 μg/mL streptomycin, and 100X non-essential amino acids. Cell numbers were assessed by Casy-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany) and adjusted to 5 X 10^6 cells/mL as described in our earlier studies (Donner et al., 1999).

Flow Cytometric Analysis: One hundred microliters of freshly isolated splenocytes or thymocytes at a concentration of 5 X 10^6 cells/mL (5 X 10^5 cells/well) were plated in Corning Cell Wells™ 96-well round-bottom tissue-culture plate (Corning, Corning, NY). Cells were washed in PBS to avoid any inadvertent stimulation from the FBS- supplemented media. Aliquots of splenocytes were dual stained with fluorescein isothiocyanate (FITC) conjugated anti-mouse CD90.2 (Thy1.2) and phycoerythrin (PE) conjugated antiCD45R/B220 antibodies (Pharmingen, San Diego, CA) or relevant isotype-matched FITC and PE anti-rat IgG$_{2a}$κ control antibodies and subjected to flow cytometric analysis by procedures reported in our earlier studies (Verthelyi and Ansar Ahmed, 1998; Ansar Ahmed et al., 1994; Ansar Ahmed and Talal, 1989; Donner et al., 1999). Thymocytes were dual stained with 100 μl of a PBS-antibody cocktail containing FITC conjugated anti-mouse CD8a and PE conjugate anti-mouse CD4 antibodies (Pharmingen, CA) or appropriate fluorochrome-tagged isotype anti-rat IgG$_{2a}$ control antibodies for 30 minutes at 4^0 C in the dark. Cells were washed in PBS and analyzed on a Coulter Epics

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XL/MXL flow cytometer (Hialeah, FL). The data were analyzed with the Immuno-4 software program (Donner et al., 1999; Ansar Ahmed et al., 1994; Karpuzoglu-Sahin et al., 2001a).

7-AAD Staining of Lymphocytes: Thymocytes were triple stained with FITC- anti-CD8 antibodies and PE-anti-CD4 antibodies and 7-aminoactinomycin D (7-AAD) as described in our previous studies (Gogal et al., 2000). One hundred microliters of unstimulated thymocytes at a concentration of 5×10^6 cells/mL (5×10^5 cells/well) were plated in Corning Cell Wells™ 96-well round-bottom tissue-culture plate (Corning, Corning, NY). Thymocytes were dual stained with anti-CD4 and anti-CD8 monoclonal antibody cocktail for 30 minutes as mentioned above. Cells were then washed in cold PBS and 100 μl of the DNA binding dye, 7-AAD (10 μg/ml; Molecular Probes, Eugene, OR) in a supplemented buffer (0.1% BSA, 0.1% NaN_3, 1.0% fetal bovine serum in PBS) was added to each well as per our previous studies (Donner et al., 1999; Gogal et al., 2000). The cells were incubated at 4^0 C for 20 minutes and then immediately analyzed by the flow cytometer. Each subset of thymocyte ($CD4^-8^-$, $CD4^+8^+$, $CD4^+8^-$, $CD4^-8^+$) was gated and the percentages of live and apoptotic cells determined by 7-AAD analyses. Staining of cells with 7-AAD allows the identification of cells at three stages of cell viability/apoptosis, these include: $7\text{-AAD}^{\text{dull}}$ (live), $7\text{-AAD}^{\text{intermediate}}$ (early apoptosis), and $7\text{-AAD}^{\text{bright}}$ (late apoptosis/necrotic) (Donner et al., 1999; Schmid et al., 1994; Gogal et al., 2000). Total apoptotic cells refer to $7\text{-AAD}^{\text{intermediate}}$ plus $7\text{-AAD}^{\text{bright}}$ cells. In our previous studies, we have shown that 7-AAD analyses closely match with FITC-annexinV and are superior to propidium iodide and forward/side scatter analysis (Donner et al., 1999; Gogal et al., 2000).

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Cytological examination of isolated splenic leukocyte fractions: Aliquots of freshly isolated splenic cells (100 µl at 5.0 x 10^5 cells) were added to chambers on the cytospin apparatus containing 0.5% BSA-PBS. Each cytospin apparatus was then placed into the Cyto-Tek centrifuge (Sakura Fine technical, Tokyo, Japan) and spun at 500 RPM for 5 min. The slide was removed from each cytospin apparatus and allowed to air dry for a minimum of 12-16 hours. Subsequently, the slides were stained with a modified Wright stain and qualitative and quantitative assessment of leukocytes done in a blinded manner. For each slide, 200 leukocytes were enumerated (lymphocytes, neutrophils, basophils, eosinophils and macrophages). In addition, cells with cytological characteristics of apoptosis, necrosis and dividing cells were quantified.

Lymphocyte Proliferation Assay: One hundred microliters of splenocytes at a concentration of 5 X 10^6 cells/mL (5 X 10^5 cells/well) were plated into each well of a Corning Cell Wells™ 96-well round-bottom tissue-culture plate (Corning, Corning, NY).

Cells were exposed to one hundred microliters of Con-A [submitogenic concentration (1µg/mL) and mitogen concentrations (5, or 10 µg/mL)], LPS (1, 5,or 10 µg/mL), or PMA (10ng/mL) and ionomycin (0.5 µg/mL) (Sigma) in complete media. Non-stimulated control cultures consisted of 100 µl complete media alone.   Triplicate wells were used for each stimulant. After 24 hours of incubation at 37^0 C in 5% CO_2, 20 µl of alamarBlue dye (Biosource International, Camars, CA) was added to each well. After 48 hours of cell incubation, fluorometric readings were recorded at wavelengths of 530 nm excitation and 590 nm emissions by CytoFluor™ II multiwell plate reader (PerSeptive Biosystems, MA). Our laboratory has extensively shown, in a wide range of species and culture systems, that the Alamar Blue assay is a reliable alternative to the

[3]H-thymidine incorporation assay (Ansar Ahmed et al., 1994; Gogal et al., 1997; Zhi-Jun et al., 1997; Saker et al., 2001).   The pattern of mitogen-induced lymphocyte proliferation assessed by the Alamar Blue assay closely matched that represented by the 3H-thymidine incorporation assay (Ahmed et al., 1994; Gogal et al., 1997; Zhi-Jun et al., 1997). The alamarBlue dye in the presence of activated/proliferating cells undergoes a chemical transformation from a non-fluorescent state to a fluorescent state. The extent of fluorescence directly correlates with proliferation. The triplicates were averaged for each stimulant for data analysis.   Cells cultured in the absence of any added mitogens (unstimulated cells) are referred to as "spontaneous proliferation".   The difference between the stimulated cultures to the non-stimulated control cultures incubated in media is referred to as specific delta relative fluorescence units.

Cytokine Analysis: Sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect IFN-γ protein levels in supernatants of cell cultures after 24 and 48 h of incubation. As described in our previous studies (Karpuzoglu-Sahin et al., 2001b), 96-well Maxisorp high-binding immunoassay plates (Fisher Scientific, Sowanee, GA) were coated with purified anti-IFN-γ clone R4-6A2 antibody (Pharmingen, San Diego, CA). Plates were blocked in 2%BSA, PBS. The assay was standardized using a serial dilution of recombinant IFN-γ (Pharmingen). Biotin conjugated anti-IFN-γ, Pharmingen overlaid with horseradish peroxidase solution (Vector Labs, Burlingame, CA) were incubated in each well. Substrate 3,3',5,5'tetra-methylbenzidine (TMB) (KPL, Gaithersburg, MD) was used to detect the colormetric assay. The plate was read at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). The IFN-γ protein levels were extrapolated using the linear region of the standard curve calculated by the SoftMax Pro Molecular Devices Inc. software.

Western Blotting: Splenocytes (2.5×10^6) from control or DES-treated mice were cultured for 24 hours in the presence or absence of 10 µg/mL of Con-A in complete media. Cells were resuspended in 15 µl of lysis buffer, (50mM Tris pH 7.4, 0.5% Triton X-100, 0.3M NaCl, 2 mM EDTA, protease inhibitor cocktail,) vortexed, and incubated on ice for 30 minutes. The samples were then centrifuged at 14,000 RPM and 4^0 C for 5 minutes to pellet DNA and cellular debris. The supernatant (cell lysate) was added to a new tube containing 15 µl of 2X Laemmli sample buffer (Sigma, Saint Louis, MO) and heated to 95^0 C for 5-10 minutes. Samples were electrophoresed on a 12.5% SDS-PAGE gel, transferred onto a Hybond-P hydrophobic polyvinylidene difluoride (PVDF) membrane, (Amersham Pharmacia Biotech, Piscataway, NJ,) and blocked in Tris Buffered Saline (TBS) with 0.1% Tween-20 and 5% non-fat dried milk for one hour at room temperature on a platform shaker. These membranes were then incubated with the appropriate primary antibody (anti-Bax, anti-Bcl-2 or anti-P55, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted in blocking buffer, overnight at 4 degrees C on a platform shaker. Membranes were then rinsed twice in TBS with 0.1% Tween-20 (TBST), and then washed for three 10 minute incubations at room temperature in TBST. Membranes were incubated with the appropriate horseradish-peroxidase conjugated secondary antibody, diluted in blocking buffer, for 1 hour at room temperature on a platform shaker. After incubation with the secondary antibody, the membrane was rinsed twice in TBST and then washed for four 10-minute incubation in TBST on a platform shaker. Membranes were developed using Enhanced Chemiluminescence (ECL) Plus reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacturer's instructions, and exposed to Hyperfilm ECL film. (Amersham Pharmacia Biotech, Piscataway, NJ.)

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Statistics: An ANOVA was performed to test for main effects of dose and gender as well as their interaction. Post-hoc mean separation was conducted using Bonferroni-corrected multiple comparisons. The MIXED procedure of the SAS System (ver. 8.1, SAS Institute Inc., Cary, NC 27513) was used to perform the calculations and P-values less than 0.05 were considered significant. For cytology counts of cells with low occurrence the analysis was modified by modeling the response as a poisson-distributed variable using the GENMOD procedure of the SAS system. Data is represented as mean ± standard error of the mean (SEM).

RESULTS

I. Effects of short-term DES exposure on weights of reproductive and lymphoid organs:

(a). Body weights and reproductive organs: Short-term administration of DES to either female or male CD1 mice did not reduce their body weights (**Table 2.1**) or social and feeding habits, suggesting that DES treatment did not have pronounced toxicity. Female mice given the high dose of DES at 30 µg/kg bw instead had a higher body weight compared to controls. This may be due to the growth promoting properties of DES, which is one of the reasons why agricultural feeds were supplemented with DES.

Administration of DES to 7-8 week-old mice, unlike in immature mice, did not appreciably affect the uterine weights (**Table 2.1**). In male mice, DES at 15 and 30 µg/kg bw significantly reduced seminal vesicular weights and the ratio of seminal vesicular to body weight. DES treatment of male mice did not effect testicular weights (**Table 2.1).**

(b). Thymic Weight: Gender differences in thymic size were observed. **Table 2.1** shows females (controls given oil-only) have significantly heavier thymuses compared to their male counterparts. Short term DES treatment did not significantly alter the thymic weight or thymic body weight ratios in either gender when compared to gender-matched control mice (**Table 2.1**). The only exception is the female mice given 30 µg/kg bw of DES which had decreased thymic to body weight ratio compared to the thymic body weight ratio of control female mice (**Table 2.1**).

(c). *Splenic weights:* Unlike the thymus, there were no gender differences in splenic weights between male and female (oil-treated) control mice. DES given to female CD-1 mice at both medium (15 µg/kg bw) and high (30 µg/kg bw) dose significantly increased splenic weight and splenic body weight ratios compared to splenic weights or splenic body weight ratios of control mice (**Table 2.1**). The splenic weight of male mice also increased in a dose related manner, although statistical significance was not achieved (**Table 2.1**).

II. Histological effects of DES on reproductive tissues:

Uteri in the control group were inactive with dense endometrial stroma. The glands were quiescent, with epithelium having scant cytoplasm (**Figure 2.1a**). Dose-dependent changes were observed in the female mice. The 5 µg/kg bw DES treatment group had relatively mild changes compared to the control group, with only a slight increase in the amount of cytoplasm in the glandular epithelial cells (**Figure 2.1b**). Uteri from the treatment group given 30 µg/kg bw of DES had very tall endometrial epithelium. The epithelium in these animals had become hyperplastic and had developed intraepithelial lumina. The endometrial glands of these

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animals had tall epithelium with prominent cytoplasm. Many of the glands contained

proteinaceous secretory material in them (**Figure 2.1d**). The uteri from the 15 µg/kg bw DES

treatment group were similar to those of the 30 µg/kg bw DES treatment group, although the

hyperplastic changes were milder and no intraepithelial lumina had developed in the

endometrial epithelium (**Figure 2.1c**). The glandular epithelium had prominent cytoplasm,

although fewer glands had secretion within them than did those in the 30 µg/kg bw treatment

group. Histological changes in the seminal vesicles of the males were minimal. The seminal

vesicular epithelium of the control group had slightly more cytoplasm, and this was marginally

more vacuolated, than the epithelium of the treatment groups. The intergrades between the

groups in amount of cytoplasm and degree of vacuolation were minimal (data not shown).

These findings are in agreement with the previous studies, which demonstrated DES-induced

histological changes in the height of the uterine epithelial cells (Mehmood et al., 2000).

III. **Short-term effects of DES on the thymus, a T-cell developmental organ:**

(a). Thymic cellular composition and alternations in apoptosis: Short-term DES-treatment did

not alter the relative percentages of $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, or $CD4^-CD8^+$ thymocyte

subsets in either female or male mice (**Table 2.2**). However, 7-AAD triple color analysis of

these subsets revealed that DES affected the viability and apoptosis. Female and male mice

given the medium and high DES dose (15 and 30 µg/kg bw) had a significant decrease in the

percentages of live ($7\text{-}AAD^{dull}$) $CD4^+CD8^+$ thymic cells and a corresponding significant

increase in late ($7\text{-}AAD^{bright}$) and total apoptotic cells [($7\text{-}AAD^{intermediate}$ (early apoptosis) plus 7-

AAD^{bright} (late apoptosis/necrotic)] (**Figure 2.2**). A similar trend of decreased live and

increased apoptotic $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were also noticed. Immature thymocytes

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(CD4⁻CD8⁻) from female mice given 30 µg/kg bw DES also had increased dead cells (**Table 2.3**).

IV. <u>Short-term administration of DES effects on splenic functions in female and male CD-1 mice.</u>

(a). DES effects on leukocyte differentials: We first determined whether *in vivo* administration of DES altered the percentages of lymphocytes and granulocytes in freshly isolated splenic lymphocytes. As can be seen in **Table 2.4,** there were no differences in the relative percentages of lymphocytes, eosinophils or basophils or macrophages. Further, in DES-treated mice, there were no marked differences in the percentages of apoptotic or necrotic or dividing cells when compared to cells from oil-treated controls.

(b).   DES effects on splenic T and B cells: DES treatment of female mice did not markedly alter the percentages of lymphocytes expressing a pan T-cell marker, CD90 , (Mean ± SE, n=5/group, Oil: 25.8 ± 1.5; DES (5 µg/kg bw): 26.4 ± 3.9; DES (15 µg/kg bw): 27.5 ± 1.1; DES (30 µg/kg bw): 19.1 ± 0.3). A similar trend was also evident in male DES-treated mice (Oil: 28.6 ± 1.3; Male: DES (5 µg/kg bw): 19.0 ± 5.0*; DES (15 µg/kg bw): 34.2 ± 2.8; Male: DES (30 µg/kg bw): 30.5 ± 6.2.).

DES treatment of female mice also did not alter lymphocytes expressing CD45R antigen which is predominantly present on B cells (and to a lesser extent on lymphokine activated cytotoxic killer cells) (Oil: 49.8 ± 1.1; DES (5 µg/kg bw): 51.3 ± 2.6; DES (15 µg/kg bw): 47.0 ± 1.2; DES (30 µg/kg bw.) 50.3 ± 1.6.).   Similar data were also seen in males given DES (data not shown).

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(c). DES effects on mitogen-induced proliferation: DES treatment of female mice did not alter spontaneous proliferation of splenic lymphocytes cultured for 48 hours (Mean Δ fluorescence units $\pm$ SE, n=6-8/group; Oil: 1652 ± 110; DES 5 µg/kg bw: 1537 ± 100; 15 µg/kg bw: 1619 ± 177, and 30 µg/kg bw: 1773 ± 116). Analogous to female mice, DES treatment of male mice also did not alter spontaneous proliferation of splenic lymphocytes (Oil: 1794 ± 229; 5 mg/kg bw: 1458 ± 153; 15 µg/kg bw: 1406 ± 172; and 30 µg/kg bw: 1550 ± 223). However, DES influenced the pattern of proliferation of splenocytes induced by mitogens, which were influenced by not only the dose of the hormone but also the gender. Splenocytes from low-dose (5 µg/kg bw) DES-treated female mice when stimulated with an optimal concentration of Con A (10 µg/mL) induced a higher level of proliferation when compared to cells from oil-only treated female controls (**Figure 2.3C**). In contrast to what was observed in mice given low dose DES, Con-A-activated splenocytes from female mice given medium or high dose of DES (15 and 30 µg/kg bw, respectively) exhibited decreased proliferative response compared to controls (**Figure 2.3C**). This pattern was not evident in age-matched male mice that were exposed to DES. Unlike in female mice, male mice treated with 5 µg/kg bw of DES had no effect on Con-A-induced lymphocyte proliferation. However, male mice treated with medium dose of DES (15 µg/kg bw) had higher level of Con-A-induced proliferation. Unlike in female DES-exposed mice, suppression of Con-A-induced proliferation in splenocytes from male mice given 30 µg/kg bw was not evident (**Figure 2.3C**).

We next examined whether splenic lymphocytes from female DES-treated mice also have a similar hormetic response when exposed to LPS. Low-dose DES (5 µg/kg) treated females had increased splenic lymphocyte proliferation in response to all concentrations of LPS (**Figure**

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2.4A, 2.4B, 2.4C). Females given higher doses of DES (15 and 30 µg/kg) did not demonstrate this enhanced ability to proliferate. DES-treated males, on the other hand, had no (to modest) increase in proliferation in response to stimulation by LPS (**Figure 2.4**).

Splenocytes from female and male DES-exposed mice when exposed to PMA plus ionomycin, displayed a pattern of lymphocyte proliferation similar to that noticed after Con-A stimulation.   In females, there was a biological trend of increased proliferation from splenocytes from low dose DES-treated mice (5 µg/kg bw) and a decrease in proliferation in mice treated with medium to higher dose of DES (**Figure 2.5**). In males, PMA-ionomycin had no effect in low dose DES-treated and no decrease in proliferation   in mice given medium or high dose of DES (**Figure 2.5**).

(d). DES induced changes in mitogen-induced proliferation of females is not due to alterations in the expression of antiapoptotic proteins: In female mice, the enhanced or suppressed Con-A-induced proliferation (noticed at 5 and 15 or 30 µg of DES/kg bw, respectively) did not correlate with the expression of anti-apoptotic, bcl-2, or apoptotic, bax, proteins (data not shown).   Similar results were also noticed in male mice given DES.

(e). DES effects on cytokines: Supernatants of Con-A activated lymphocytes from female CD-1 mice tended to have higher levels of IFN-γ compared to their male counterparts. The supernatants of Con-A- stimulated splenic cells from female mice given DES tended to have a decreased level of IFN-γ protein and significance observed in cultures from mice given 5 µg /kg bw) (**Figure 2.6**). Interestingly, IFN-γ did appear to be enhanced as the DES dose increased. In males, supernatants of Con-A-activated splenocytes from mice treated with all doses of DES

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tended to have lower levels of IFN-γ, although not statistically significant (**Figure 2.6**). DES did not markedly alter levels of IL-2 or IL-4 (data not shown).

DISCUSSION

Two questions relative to the immunomodulatory effects of DES are thus far not well known and hence are addressed in the present studies. First, does the immunomodulatory effects of DES vary with the dose of the hormone and second, are these effects seen in both gender? These questions are clinically important since both genders were exposed to DES at a large range of dose because a standard dose regimen was not firmly established. It is also pertinent to note that the dose of DES employed in experimental animals also varied largely among studies. The present report indicates that immunological effects of DES vary with the dose of the hormone and the gender.

A noteworthy anatomic observation was that the thymuses from female oil-treated control CD-1 mice were heavier than their male counterparts. Similar results were also noted in NMRI mice (Forsberg, 2000). The precise reasons for this gender difference are not apparent, but it may be due to differential response to endogenous gonadal and/or corticosteroid hormones, which are known to affect the thymic size. Despite these gender differences in thymic weights, both males and females had similar percentages of thymocyte subsets.

Short-term DES treatment of either females or males did not induce thymic atrophy as evidenced by unchanged relative thymic weights or thymic to body weight ratios. The only exception was a decrease in thymic: body weight ratio in female mice given 30 μg/kg bw of DES. This may be a reflection of increase in body weight rather than an effect on the thymus,

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since at this dose, DES increased the body weight but had no affect on the weight of the thymuses. Short-term DES treatment also did not alter the relative percentages of $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ thymocyte subsets. Other studies have shown that the DES treatment of neonates or aged mice had adverse effects on the thymus and/or decreased percentages of $CD4^+CD8^+$ thymocyte subsets (Forsberg, 1984; Hirahara et al., 1994; Endo et al., 1994; Rijhsinghani et al., 1996; Holladay et al., 1993; Smith and Holladay, 1997). The absence of thymic atrophy in DES-treated mice in the present studies may be due to differences in the mouse strain (outbred CD-1 mice compared to inbred mouse strains used by others) and the duration of the study. Further, recent studies have suggested that the long-term consequence of DES effects on the thymus varied with the age when exposure occurred. For example, in neonatal DES-treated NMRI mice (5 injections of 5 μg of DES on days 1-5 of age), there was a thymic enlargement by 8 weeks after DES treatment. Despite the enlarged thymus, there were no alterations in the relative percentages of $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocyte subsets (Forsberg, 2000). In mice treated with DES during post-pubertal life (48-52 days of age), there was no thymic enlargement but the gland was comparable to controls (Forsberg, 1996; Forsberg, 2000). Although our studies suggested that short-term DES did not induce thymic atrophy in 8-week-old CD-1 mice, by triple color flow cytometric analysis, we noticed that DES induced cell death of $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocyte subsets. Analysis of these subsets revealed that there was a dose-related increase in 7-AAD positive cells. We and others have shown that 7-AAD^{intermediate} cells correspond to early apoptotic Annexin-V$^+$ cells, while 7-AAD^{bright} cells represent late apoptotic or necrotic cells (Donner et al., 1999; Schmid et al., 1994). It is possible that this DES-induced cell death of thymocyte subsets may represent a prethymic atrophy stage. In our previous *in-vitro* studies, by multiple techniques we

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have shown that the direct exposure of isolated thymocytes from mature C57BL6 mice to DES did not induce apoptosis (Donner et al., 1999). A recent study by Lai et al. (2000) who employed developing thymus (which has a large subset of CD4$^-$CD8$^-$ cells, unlike in mature thymus) in a fetal thymic organ culture system (which also has intact stromal populations), have shown that DES induced apoptosis of CD4$^-$CD8$^-$ subset. Adverse effects of DES on thymus could be due to the following possibilities. First, apoptosis of thymocyte subset(s), which may be due to indirect untoward effects on thymic stromal and nurse cells that provide nurturing cytokines and signals that are necessary for thymocyte survival. Although less likely, direct apoptotic effects of DES on thymocyte subsets remains a possibility. Second, blockage of CD4$^-$CD8$^-$ cells in G2/M stage of cell cycle (Lai et al., 2000) or third, decreased immigration of prothymocyte stem cells (Holladay et al., 1993).

Female, but not male, mice given DES at medium and high doses had significantly increased splenic weights (a similar trend was also evident in DES-treated males). Similar findings have been observed in neonatal DES-treated NMRI mice when examined 8 weeks after DES treatment (Forsberg, 2000). This increased in splenic weights could not be attributed to increase in relative percentages of CD90$^+$ or CD45R$^+$ cells, suggesting that DES did not alter T or B cell percentages. Further, the numbers of lymphocytes, macrophages, basophils and eosinophils were also not increased. In female mice given DES, there appeared to be a modest increase in neutrophils. The precise reasons are not apparent. It is possible that DES may affect chemokines or chemotactic factors that recruit neutrophils from bone marrow or other sites. Although DES did not alter the percentages of T or B cells, it did affect the functionality of splenocytes. Female mice treated with low dose of DES had a pattern of increased mitogen-induced proliferation of splenocytes. This type of response curve was not evident in age-matched

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males given the same dose of DES. It is not clear if this is due to an additive effect of DES and endogenous female sex hormones or whether male hormones counter the effect of DES. Future studies should address this issue by performing similar experiments in gonadectomized mice. In contrast to the enhanced mitogen-induced proliferative response of splenocytes from mice given 5 µg/kg bw of DES, similar cultures from mice given a higher dose of DES (30 µg/kg bw) did not demonstrate an enhanced proliferative response. Rather, lymphocyte proliferation was decreased. To our knowledge, this is the first study to show the hormetic immunological responses with low dose of DES having a stimulatory effect, while high doses eliciting an inhibitory effect, a phenomenon previously noticed in endocrine tissues (Atanassova et al., 2000; vom Saal et al., 1997). In males, given higher doses of DES there was no suppression of mitogen-induced lymphocyte proliferation. These alterations are not the consequence of altered numbers of T or B cells, since their relative percentages were not affected by the short term DES treatment. Our results also suggest that alterations of mitogen-induced lymphocyte proliferation in DES-treated mice are not due to concomitant changes in the ratios of proapoptotic (bax) to anti-apoptotic (bcl-2) proteins. Nevertheless, it is still possible that DES affected activation-induced apoptosis by pathways other than bcl-2 and bax (example, caspase independent pathway, Fas-FasL, bcl-XL, bad, bid etc), an aspect beyond the scope of this study. We also examined whether theses differences in mitogen-induced proliferation are due to alterations in the expression of p55CDC protein, which is involved in proliferation/cell cycle, by using anti-p55CDC antibody (Santa Cruz Biotech Inc., CA) in a Western blot analysis. The pattern of proliferation could not be correlated with the presence of this protein (data not shown). It is possible that DES-induced alterations in mitogen-induced lymphocyte proliferation may be due

to changes in T cell receptor or co-stimulatory-mediated signaling, suppressor/regulatory cytokines, or progression in cell cycle an aspect currently being investigated by separate studies.

In the present study, we find that supernatants of Con-A activated splenic lymphocytes from female mice had higher levels of IFN-γ (4270 $\pm$ 144.4 pg/mL) compared to their male counterparts (3289 $\pm$ 200 pg/mL). A similar trend in gender differences was noticed in our recent study (Karpuzoglu-Sahin et al., 2001b). DES administration, especially in females, tended to decrease the levels of IFN-γ. This further supports that DES functionally alters lymphocytes. We have recently shown that 17β estradiol also regulated the levels of IFN-γ (Karpuzoglu-Sahin et al., 2001b). Further, the levels of IFN-γ were altered in C57BL/6 mice given DES during prenatal and subsequently in adult life (Karpuzoglu-Sahin et al., 2001a). In these studies, we noticed that estrogenic hormones promoted IFN-γ levels, this may be due to differences in strain, experimental system and the higher dose of DES. Regardless, relevant to this study, our findings suggest that estrogenic hormones regulate IFN-γ levels. Together our studies suggest that DES effects on the immune system are dependent upon the dose of the hormone and the gender of the recipient.

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Figure 1
A. DES effects on endometrial epithelial cell height
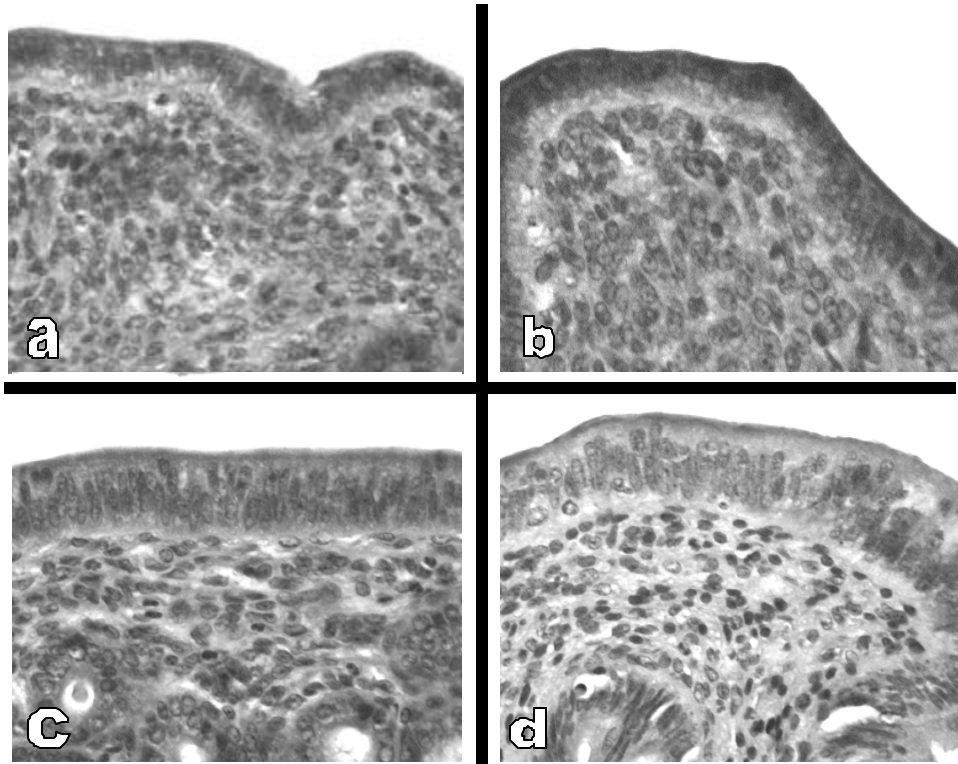
B. Mean data of DES effects on endometrial epithelial cell height

	DES (µg/kg bw)			
	0	5	15	30
Epithelial Cell Height (μm $\pm$ SEM)	4.29 ± 0.33	4.58 ± 0.15	**6.17 ± 0.60****	**6.17 ± 0.54****

** = p<0.005

Figure 2.1: *DES induces histological alterations in the reproductive tract:* Uteri from DES and control–treated mice were fixed in 10 % buffered formalin and subjected to histopathological staining. (A) shows representative histopathological sections from the uteri of 0, 5, 15, 30 µg/kg bw DES-treated mice. Note mice treated with DES, especially at 15, 30 µg/kg bw dose levels, had tall epithelial cells. (B) shows mean morphometric data of the uteri epithelial cell height (in µm) ± standard error of the mean (SEM) from DES and oil treated mice (n=4 per each treatment group). The statistical significance (*****p<0.05) between DES-treated and oil-control groups was determined as described in the methods section.

Figure 2

A. DES-exposure Increases Apoptosis of CD4$^+$CD8$^+$ Thymocytes

Oil

CD4 / CD8 — 66.5%

Oil (CD4$^+$CD8$^+$)

Late apoptosis — 8.7%
Early apoptosis — 4.1%
Live — 87.2%

7-AAD / Forward Scatter (0 – 1024)

DES- 30µg/kg bw

CD4 / CD8 — 65.8%

DES- 30µg/kg bw (CD4$^+$CD8$^+$)

Late apoptosis — 22.6%
Early apoptosis — 3.7%
Live — 73.6%

7-AAD / Forward Scatter (0 – 1024)
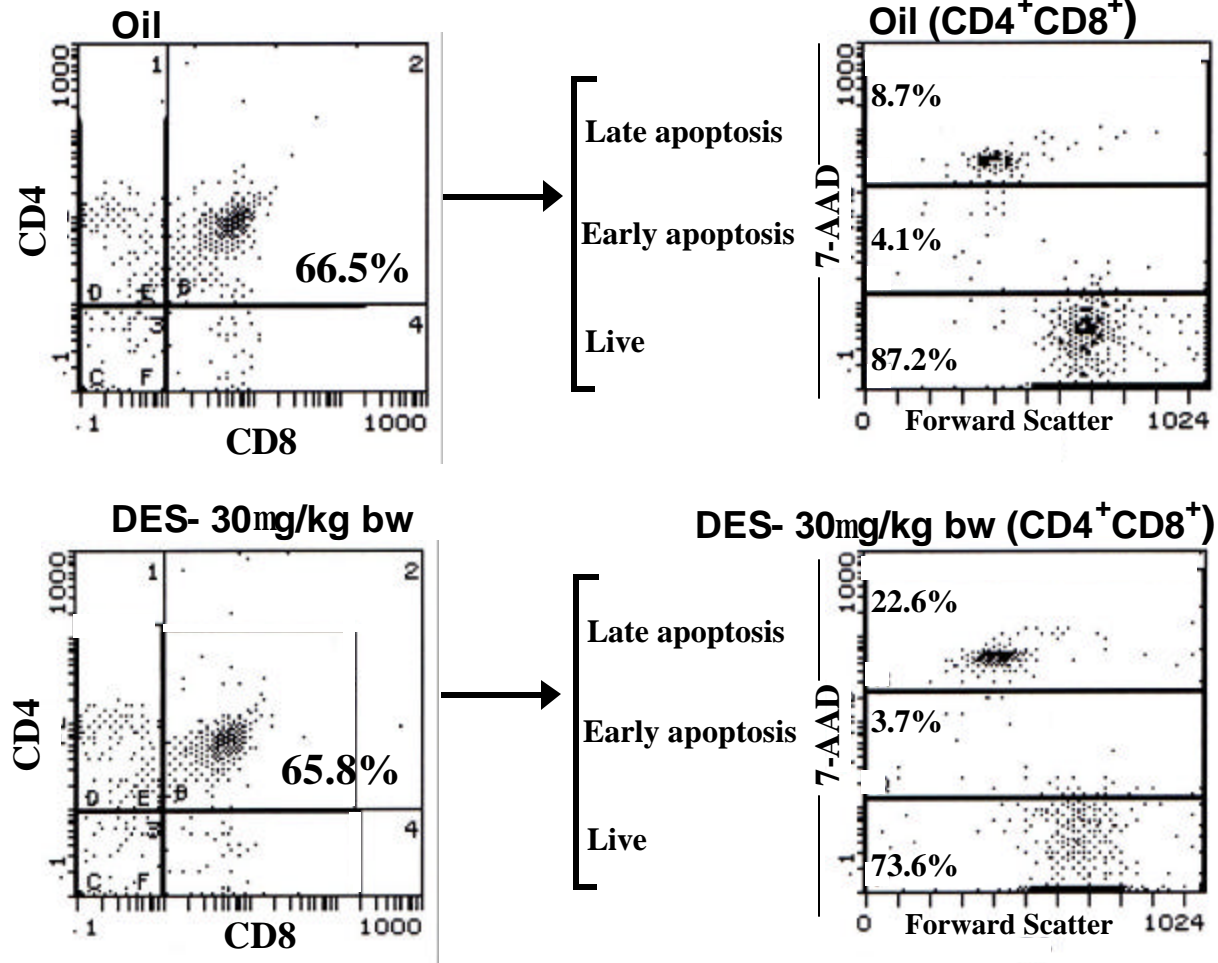
B. DES-exposure Increases Apoptosis of CD4$^+$CD8$^+$ Thymocytes: Mean Data ± SEM

	Females				Males			
	DES (µg/kg bw)				DES (µg/kg bw)			
	0	5	15	30	0	5	15	30
Live cells	87.7 ± 0.8	83.5 ± 1.6	**80.3 ± 3.9***	**76.1 ± 5.1***	90.3 ± 2.1	88.0 ± 0.9	**83.7 ± 1.8***	**81.3 ± 1.6***
Early Apoptotic cells	3.6 ± 0.3	3.7 ± 0.4	3.1 ± 0.3	3.4 ± 0.5	5.0 ± 0.7	**6.8 ± 0.5***	5.3 ± 0.3	4.7 ± 0.4
Late Apoptotic cells	8.7 ± 0.8	12.8 ± 1.3	**16.6 ± 4.1***	**20.5 ± 4.9***	4.7 ± 1.7	5.4 ± 0.6	**11.1 ± 1.9***	**14.0 ± 1.9***
Total Apoptotic cells	12.3 ± 0.8	16.5 ± 1.6	**19.7 ± 3.9***	**23.9 ± 5.1***	9.7 ± 2.1	12.2 ± 0.9	**16.4 ± 1.8***	**18.7 ± 1.6***

* $p < 0.05$ compared to gender matched control

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Figure 2.2: *Triple color 7-AAD staining of oil and DES treated female thymocytes*: Female and male mice were treated with four injections of the indicated doses of DES as described in the legend of table 1. Lymphocytes from DES-treated mice were triple stained with PE-anti-CD4 antibodies, FITC-anti-CD8 antibodies and 7-AAD. Control cultures were stained with fluorochrome –tagged PE and FITC-isotype matched antibodies and unstained (third color control for 7-AAD). $CD4^+CD8^+$ thymocytes were gated and analyzed for 7-AAD staining. This DNA-binding dye identifies three distinct subsets, 7-AAD^{dull} or live, 7-AAD^{intermediate} or early apoptotic and 7-AAD^{bright} late apoptotic/necrotic. Significance between treatment groups was determined by $*P<0.05$ when compared to the gender matched control. **(A)** Shows a representative data from oil and DES (30 µg/kg bw)-treated mice. This data shows that short-term DES treatment did not alter the relative percentages of $CD4^+CD8^+$ thymocytes, but did induce cell death of this subset as analyzed by 7-AAD staining. **(B)** Shows the mean data ± standard error of the mean (SEM) pertaining to the apoptosis of CD4+CD8+ thymocytes from female (n =4/ group) and male (n = 5/group) treated mice with various doses of DES. The statistical significance (**$p<0.05$) of DES treatment was assessed by comparing to their gender matched controls.
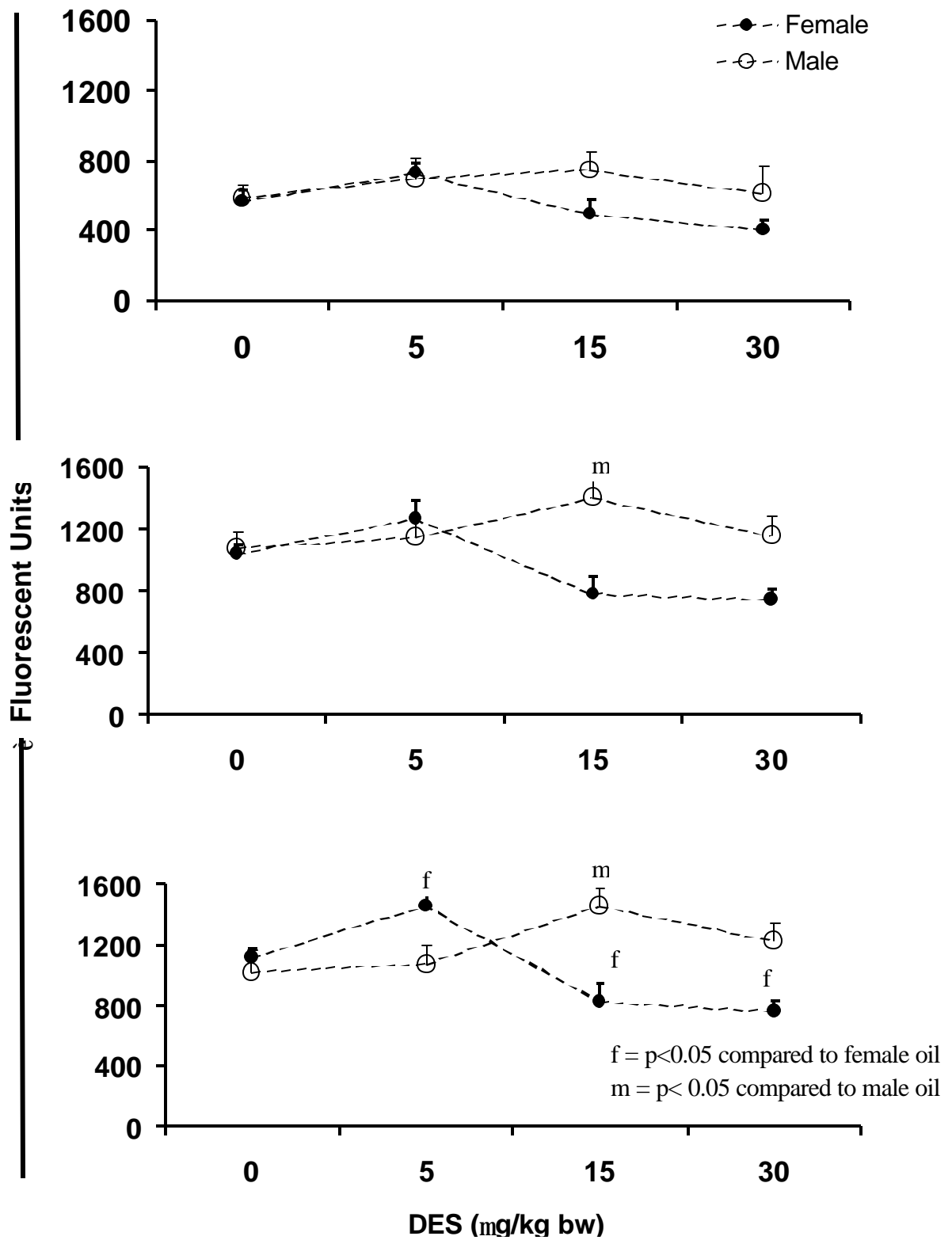
Figure 2.3: *Different patterns of Con-A-induced splenic lymphocyte proliferation between female and male mice.* Splenic lymphocytes (2.5×10^6 cells/mL) from female (n=8/ group) and male (n= 9/group)

mice treated with indicated doses of DES were stimulated with Con-A 1, 5 or 10 µg/mL (**A**, **B**, **C** panels respectively) for 48 hours. Unstimulated cells were considered as culture controls. All cultures were done in triplicates. After 24 hours of culture, 20 µL of the indicator dye, alamarBlue™ was added to all cultures. This dye, in the environment of proliferating and activated cells undergoes a chemical transformation and becomes fluorescent, which is detected by using 530 nm (excitation) and 590 nm (emission) wave length filters in a Cytofluor ™ II flurometer.   Proliferation was assessed at the end of 48 hours of culture. Data plotted are a representative of means with error bars indicating standard error of the mean. The significance between DES-treated groups and oil-treated control ($p<0.05$) was determined. The symbol "f" depicts the statistical significance of DES-treated female mice compared to oil-treated female control mice.   The symbol " m " depicts statistical significance of DES-treated male mice compared to oil-treated male control.
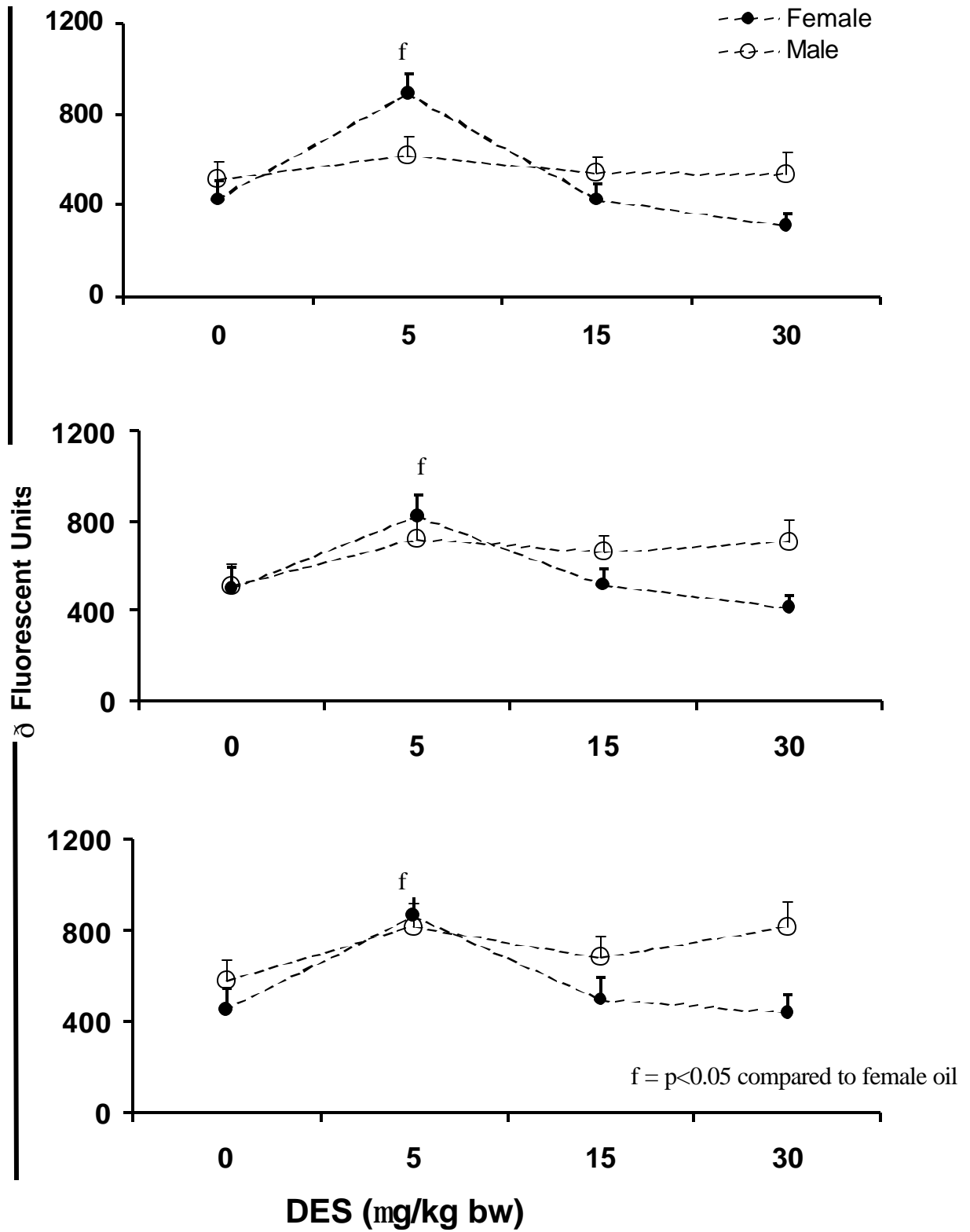
Figure 2.4: *Different patterns of LPS-induced splenic lymphocyte proliferation between female and male mice.* Splenic lymphocytes (2.5 x 10⁶cells/mL) from female (n=8/ group) and male (n= 9/group)

mice treated with the indicated doses of DES were stimulated with LPS 1, 5 or 10 μg/mL (**A**, **B**, **C** panels respectively) for 48 hours. Unstimulated cells were considered as culture controls. All cultures were done in triplicates. After 24 hours of culture, 20 μL of the indicator dye, alamarBlue™ was added to all cultures. Proliferation was assessed at the end of 48 hours of culture. Data plotted is representative of means with error bars indicating standard error of the mean. The significance between DES-treated groups and oil-treated control ($p<0.05$) was determined. The symbol "f" depicts the statistical significance of DES-treated female mice compared to oil-treated female control mice.
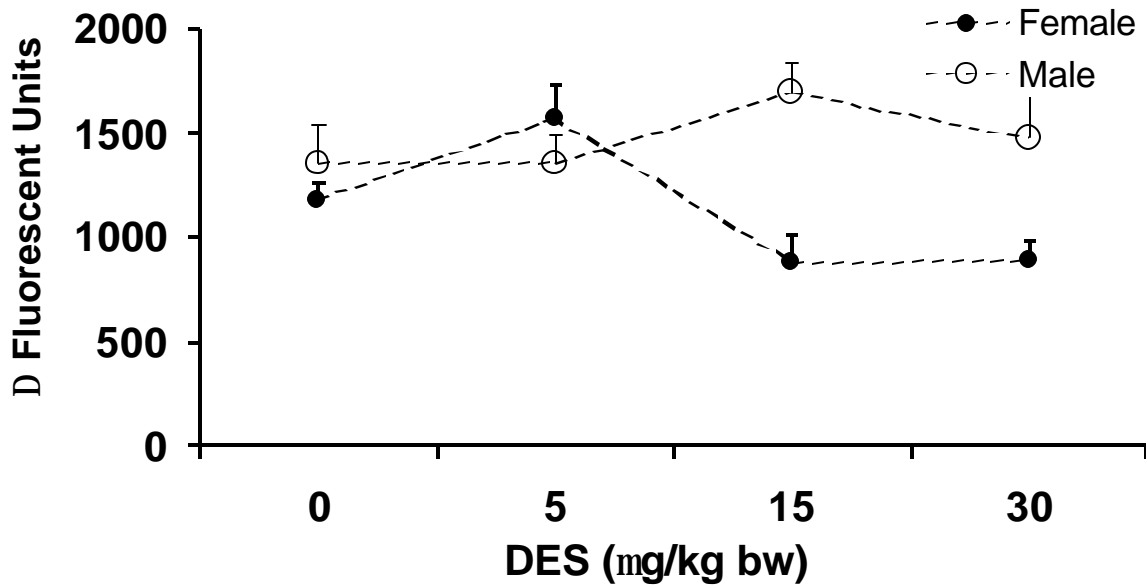
Figure 2.5: *Different patterns of PMA-ionomycin-induced splenic lymphocyte proliferation between female and male mice.* Splenic lymphocytes (2.5 x 10^6 cells/mL) from female (n=8/ group) and male (n= 9/group) mice treated with the indicated doses of DES were stimulated with PMA (10 ng/mL) plus ionomycin (0.5 mg/mL) for 48 hours. Unstimulated cells were considered as culture controls. All cultures were done in triplicates. After 24 hours of culture, 20 µL of the indicator dye, almarBlue™, was added to all cultures. Proliferation was assessed at the end of 48 hours of culture. Data plotted is representative of means with error bars indicating standard error of the mean.
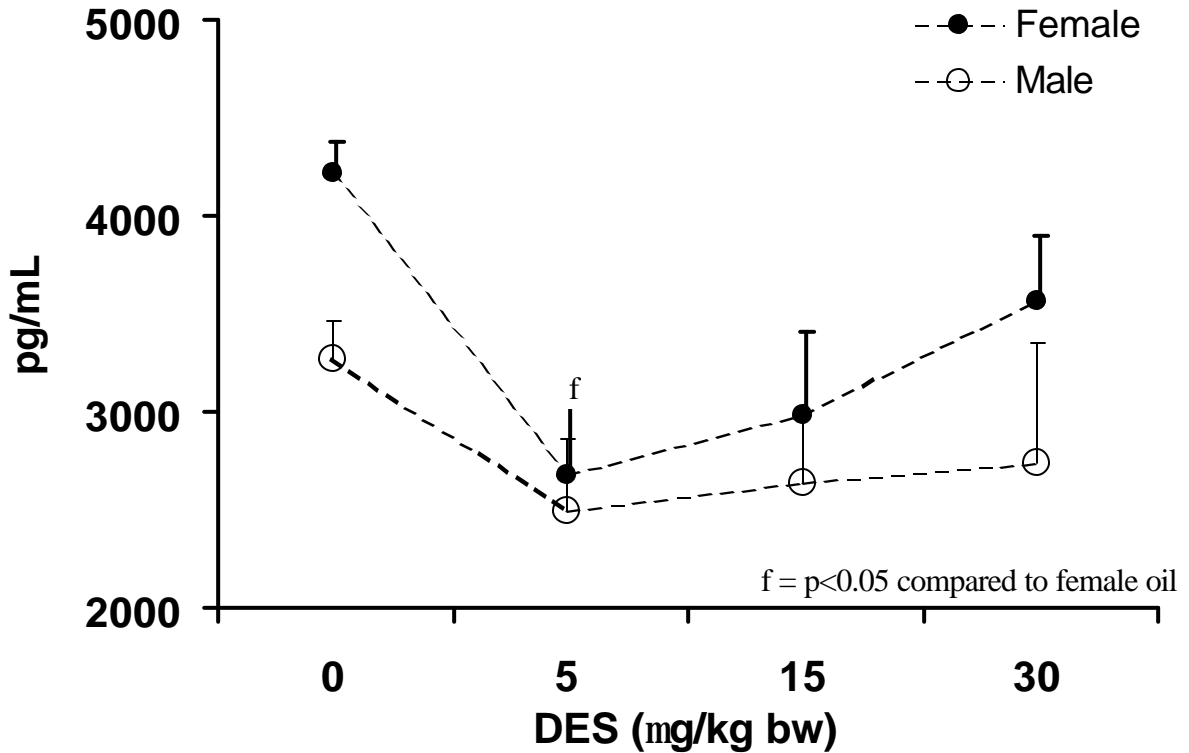
Figure 2.6: *DES affects the level of interferon-g.* Female (n= 8/ group) and male (n= 9/ group) mice were treated with the indicated doses of DES. Splenic lymphocytes from these mice were stimulated with Con-A (10 μg/mL) and the supernatants collected at 24h for analysis of interferon-γ by a specific sandwich ELISA. Positive controls included a serial dilution of recombinant interferon-γ to generate a standard curve. The amount of interferon-γ in the test samples were extrapolated from the standard curve and the data is represented as the mean ± standard error of the mean in pg/mL. The significance between DES-treated groups and oil-treated control ($p<0.05$) was determined. The symbol "f" depicts the statistical significance of DES-treated female mice compared to oil-treated female control mice.

Table 2.1

Body weights and organ weights of female and male CD-1 mice given various doses of DES

		DES (µg/kg bw)			
Organ	Gender	0	5	15	30
Terminal Body Weight	Female	25.537 ± 0.638	26.875 ± 0.809	26.388 ± 0.487	**27.975 ± 0.652***
	Male	36.555 ± 0.596	37.344 ± 0.611	35.933 ± 0.428	37.155 ± 1.080
Reproductive Organs					
Uterus					
Weight (g ± SEM)	Female	0.178 ± 0.040	0.149 ± 0.016	0.199 ± 0.030	0.152 ± 0.009
Uterine Wt. ÷ bw ± SEM [a]		0.498 ± 0.115	0.398 ± 0.041	0.521 ± 0.077	0.419 ± 0.024
Seminal Vesicle (S.V.)					
Weight (g ± SEM)	Male	0.231 ± 0.017	0.214 ± 0.018	**0.182 ± 0.013***	**0.189 ± 0.023***
S.V. Wt. ÷ bw ± SEM		0.631 ± 0.046	0.570 ± 0.045	**0.508 ± 0.037***	**0.508 ± 0.060***
Testes					
Weight (g ± SEM)	Male	0.218 ± 0.016	0.222 ± 0.020	0.200 ± 0.013	0.216 ± 0.007
Testes Wt. ÷ bw ± SEM		0.601 ± 0.050	0.596 ± 0.053	0.556 ± 0.034	0.586 ± 0.028
Lymphoid Organs					
Thymus					
Weight (g ± SEM)	Female	0.060 ± 0.002	0.064 ± 0.004	0.059 ± 0.005	0.053 ± 0.004
	Male	0.045 ± 0.004	0.052 ± 0.005	0.042 ± 0.002	0.053 ± 0.005
Thymic Wt. ÷ bw ± SEM	Female	0.237 ± 0.013	0.240 ± 0.013	0.221 ± 0.019	**0.192 ± 0.016***
	Male	0.126 ± 0.013	0.140 ± 0.015	0.117 ± 0.007	0.143 ± 0.014
Spleen					
Weight (g ± SEM)	Female	0.093 ± 0.005	0.112 ± 0.008	**0.128 ± 0.005***	**0.129 ± 0.006***
	Male	0.103 ± 0.005	0.104 ± 0.005	0.110 ± 0.008	0.122 ± 0.009
Splenic Wt. ÷ bw ± SEM	Female	0.365 ± 0.022	0.416 ± 0.022	**0.487 ± 0.014***	**0.463 ± 0.019***
	Male	0.285 ± 0.017	0.278 ± 0.012	0.307 ± 0.025	0.329 ± 0.026

[a] Uterine weight : body weight (bw) ratio

Table 2.1 (continued): *DES effects on body and organ weights:* Seven to eight - week old female (n=8) and male (n=9) were given four subcutaneous injections of DES in 50 µl of corn oil (5, 15, 30 µg/kg bw) over a one-week period. Control mice were given equivalent volumes of oil only. Mice were weighed prior to the initiation of the treatment and a day prior to the day of termination. Uteri, seminal vesicles, testes, thymuses, and spleens were weighed and these weights were also related to their body weight. Each of the DES–treated group was compared with its gender-matched oil controls. Data are presented as mean ± standard error of the mean. The significance between treatment groups (*$p<0.05$) was determined by comparing to the gender matched control.

Table 2.2

Mean relative expression of thymocyte subsets from female and male CD-1 mice given various doses of DES

	Females DES (µg/kg bw)				Males DES (µg/kg bw)			
	0	5	15	30	0	5	15	30
CD4$^+$CD8$^-$	13.5 ± 1.3	14.9 ± 1.4	15.8 ± 1.3	15.0 ± 1.2	15.9 ± 1.3	15.2 ± 2.6	15.4 ± 1.5	14.7 ± 2.3
CD4$^-$CD8$^+$	3.0 ± 0.4	3.7 ± 0.4	2.4 ± 0.2	2.8 ± 0.2	3.7 ± 0.2	5.0 ± 1.7	4.1 ± 0.4	3.0 ± 0.5
CD4$^+$CD8$^+$	69.0 ± 3.0	65.6 ± 5.8	70.9 ± 2.1	70.5 ± 2.6	62.7 ± 3.8	59.6 ± 5.8	57.7 ± 4.0	60.5 ± 4.7
CD4$^-$CD8$^-$	14.2 ± 4.2	15.9 ± 5.1	10.9 ± 2.1	11.7 ± 2.1	17.7 ± 2.7	20.1 ± 2.3	22.7 ± 5.3	21.8 ± 3.8

Table 2.2: *DES treatment and thymocyte subsets*: Seven to eight- week old male (n = 5) and female (n =4) CD-1 mice were treated with DES or oil only as described in the legend of table 1. Thymocytes from these mice were stained with PE-anti-CD4 and FITC-anti-CD8 antibodies. Flow cytometric controls included cells stained with PE and FITC-isotype matched control antibodies. The data was analyzed by immunosoftware in a Coulter Epics-XL/MXL flow cytometer. There were no marked differences in the thymocyte subsets between the oil-treated female control mice and oil-treated male control mice. Interestingly, males given DES, tended to have a higher percentages of CD4$^-$8$^-$ thymocyte subsets and a lower CD4$^+$8$^+$ thymocyte subset compared to DES-treated female mice. The data are presented as mean ± standard error of the mean.

Table 2.3

Effects of DES exposure on apoptosis of CD4$^+$CD8$^-$, CD4$^-$CD8$^+$, and CD4$^-$CD8$^-$ thymocyte subsets

CD4$^+$CD8$^-$

	Females DES (µg/kg bw)				Males DES (µg/kg bw)			
	0	5	15	30	0	5	15	30
Live cells	83.7 ± 1.7	81.8 ± 1.2	79.2 ± 1.1	**73.2 ± 3.6***	87.2 ± 2.9	85.2 ± 1.5	81.4 ± 2.3	**78.9 ± 2.4***
Early Apoptotic cells	5.4 ± 0.6	5.7 ± 0.6	6.2 ± 0.9	5.8 ± 0.8	6.5 ± 0.9	9.1 ± 1.0*	8.2 ± 0.6	7.8 ± 0.9
Late Apoptotic cells	10.9 ± 1.5	12.6 ± 0.7	14.6 ± 1.6	**21.0 ± 3.3***	6.3 ± 2.1	5.7 ± 0.7	10.4 ± 2.5	**13.3 ± 2.1***
Total Apoptotic cells	16.3 ± 1.7	18.3 ± 1.2	20.8 ± 1.1	**26.8 ± 3.6***	12.8 ± 2.9	14.8 ± 1.5	18.7 ± 2.3	**21.1 ± 2.4***

CD4$^-$CD8$^+$

	Females DES (µg/kg bw)				Males DES (µg/kg bw)			
	0	5	15	30	0	5	15	30
Live cells	88.4 ± 1.7	85.9 ± 2.2	85.0 ± 3.4	**79.4 ± 3.3***	89.4 ± 2.7	90.0 ± 2.0	87.1 ± 2.0	84.7 ± 3.1
Early Apoptotic cells	5.0 ± 1.1	5.5 ± 1.4	5.3 ± 1.3	5.0 ± 1.3	6.1 ± 1.7	6.2 ± 1.1	7.0 ± 1.0	6.5 ± 2.1
Late Apoptotic cells	6.6 ± 0.8	8.5 ± 1.1	9.7 ± 2.2	**15.6 ± 2.3***	4.5 ± 1.1	3.8 ± 1.2	5.9 ± 1.4	**8.8 ± 1.6***
Total Apoptotic cells	11.6 ± 1.7	14.1 ± 2.2	15.0 ± 3.5	**20.6 ± 3.3***	10.6 ± 2.7	10.0 ± 2.0	12.9 ± 2.0	15.3 ± 3.1

CD4$^-$CD8$^-$

	Females DES (µg/kg bw)				Males DES (µg/kg bw)			
	0	5	15	30	0	5	15	30
Live cells	87.7 ± 2.4	87.3 ± 2.7	86.4 ± 2.1	84.4 ± 1.6	92.8 ± 2.2	89.3 ± 1.6	91.1 ± 2.0	91.3 ± 2.2
Early Apoptotic cells	10.5 ± 2.2	10.7 ± 2.2	10.6 ± 1.1	10.2 ± 1.3	6.5 ± 1.8	10.2 ± 1.5	7.4 ± 1.3	7.5 ± 1.8
Late Apoptotic cells	1.7 ± 0.4	2.0 ± 0.5	3.0 ± 1.0	**5.4 ± 0.5***	0.7 ± 0.5	0.6 ± 0.2	1.5 ± 1.1	1.2 ± 0.6
Total Apoptotic cells	12.3 ± 2.4	12.6 ± 2.7	13.6 ± 2.0	15.6 ± 1.6	7.2 ± 2.2	10.7 ± 1.6	8.9 ± 2.0	8.7 ± 2.2

Table 2.3 (continued): *Effects of DES on thymocyte apoptosis*: Female and male CD-1 mice were given four injections of DES as stated in the legend of table 1. Thymocytes were triple stained with PE-anti-CD4, FITC-anti-CD8 and 7-AAD as described in the methods section. CD4$^+$CD8$^-$, CD4$^-$CD8$^+$ and CD4$^-$CD8$^-$ thymocyte subpopulations were identified and gated. Each gated thymocyte subset was analyzed for staining intensity with7-AAD against forward scatter. The data are presented as mean ± standard of the mean. Significance between treatment groups (*$p < 0.05$) was determined by comparing to the gender matched control.

Table 2.4

Cytological assessment of DES effects on splenic leukocytes

	Females DES (µg/kg bw)				Males DES (µg/kg bw)			
	0	5	15	30	0	5	15	30
Lymphocytes	95.3 ± 1.4	90.0 ± 2.4	91.8 ± 2.1	90.0 ± 1.7	88.5 ± 1.9	90.0 ± 0.8	87.0 ± 2.3	90.8 ± 1.2
Monocytes	3.8 ± 0.3	4.5 ± 1.2	4.5 ± 1.8	3.5 ± 0.6	5.0 ± 1.1	4.0 ± 1.1	7.6 ± 2.7	4.2 ± 1.2
Neutrophils	0	3.2 ± 1.3*	0.8 ± 0.3	3.3 ± 0.5*	5.8 ± 1.8	3.4 ± 0.7	3.4 ± 1.1	2.8 ± 0.8
Basophils	0	0	0	0	0	0	0	0
Eosinophils	0	0	0	0	0	0	0	0
Apoptotic	1.3 ± 0.3	1.0 ± 0.7	2.8 ± 0.6	2.8 ± 1.5	1.0 ± 0.7	1.8 ± 0.9	1.8 ± 0.8	0.6 ± 0.2
Necrotic Cells	0	0	1.3 ± 1.3	0	7.0 ± 2.9	10.8 ± 2.3	3.6 ± 0.5	4.4 ± 0.7
Dividing Cells	0	0	0.8 ± 0.5	0	0.3 ± 0.3	1.8 ±1.0	3.6 ± 2.9	3.4 ± 0.5

Table 2.4: *Effects of DES on splenic leukocyte differentials*: Freshly-isolated splenic lymphocytes from oil or DES-treated mice were subjected to cytospin, stained and cytologically evaluated in a coded fashion. The data are shown as percent mean ± standard error of the mean. Significance between treatment groups was determined by * $p<0.05$ when compared to the gender matched control.

Chapter 3
Prenatal Exposure to Diethylstilbestrol Has Long-Lasting Immunological Effects on the Thymus

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Abstract

It is well known that estrogens and estrogenic compounds target the thymus, a primary T cell developmental lymphoid organ. Natural estrogen (17β-estradiol) and synthetic estrogens (e.g. diethylstilbestrol or DES), even after a short-term exposure in neonate and adult mice, have been shown to induce thymic atrophy. Exposure to immunomodulatory DES during the critical fetal developmental period may have long-term immunological consequences. In order to study the effects of fetal exposure to estrogenic compounds on the immune system later in life, we administered DES to pregnant C57BL/6 mice and looked at the effects of DES on the thymus of offspring examined as late as one year of age. We find that prenatal DES exposure had marked effects on the thymus that were gender-specific. In females, there was a significant decrease in thymic weight and thymocyte numbers. Prenatal DES treatment in females targeted $CD4^+CD8^+$ cells. DES-treated females had a significant decrease in the relative percentage of live $CD4^+CD8^+$ cells compared to oil controls after culturing in media for 24 hours. When thymocytes were cultured in dexamethasone, which induces cellular apoptosis, only DES treated female $CD4^+CD8^+$ cells were not affected. In contrast, males prenatally exposed to DES had an enlarged thymus and an increasing trend in thymocyte cellularity. Thus, results from this study suggest that prenatal DES exposure resulted in an intrinsic defect in the thymus that is life-long. We also investigated whether prenatal exposure to DES would influence autoimmune related T-cell populations, $TCRV\beta8^+$ and $CD4^+CD25^+$ and found DES treatment had no effect on these cell types in the thymus.

Introduction

An important topic in research today is understanding how the *in-utero* environment can influence the state of wellness of the offspring later in life. Specifically, scientists are interested in learning which compounds are beneficial to the fetal development and which are detrimental. One category of environmental compounds of scientific interest is the estrogenic compounds. Estrogenic compounds, which are inherently disruptive to the endocrine system and immune system, can be found practically in every environment. These include phytoestrogens, synthetic estrogens (used in the production of plastics and pesticides) and therapeutic estrogens as well as their metabolic byproducts. One of the most potent estrogenic compounds, diethylstilbestrol, was primarily given to women during pregnancy. Once it was discovered that DES was causing severe reproductive abnormalities, it was banned from clinical use. By the time a decision was made to prohibit the use of DES in pregnant women, nearly 5 million pregnant women were exposed to DES and millions more were exposed *in-utero* (Marselos and Tomatis, 1992). The detrimental effects of prenatal exposure to DES on the reproductive system have been well documented (Herbst et al., 1971, Newbold and McLachlan, 1982, Wingard and Turiel, 1988, Golden et al., 1998). However, the mechanism(s) by which DES influences the development of the fetal immune system and its consequential effects later in life have not been thoroughly explored.

Like natural estrogen, 17β-estradiol, DES has been noted to induce thymic atrophy in mice when exposed for a short time during neonate and adult ages (Hirahara et al.,

1994, Forsberg, 1984). DES has been reported to decrease prothymocyte stem cells (Holladay et al., 1993), decrease double positive $CD4^+CD8^+$ cells (Smith and Holladay, 1997), as well as induce cell death in thymocyte subsets $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ (Calemine et al., 2002). DES has also been shown to induce apoptosis of double negative $CD4^-CD8^-$ cells in a fetal thymic organ culture system (Lai et al., 2000).

Clinical studies have shown that women who were prenatally exposed to DES were more likely to develop a rare vaginal cancer, clear cell adenocarcinoma (Herbst et al., 1971, Newbold et al., 1990). Importantly, the malignancy would not develop until the exposed person was of puberty age. Together these studies imply that prenatal DES exposure (the first "immunomodulatory trigger") will sensitize the individual and abnormalities (e.g. vaginal adenocarcinoma) will only become evident after exposure to endogenous sex hormones such as estrogen. It is possible that prior exposures to DES as early in prenatal life will "permanently" render these cells susceptible to aberrant response to subsequent exposure to estrogenic compounds. Similar results were also seen in animal studies. Secondary transplantation studies in Syrian hamsters have shown that the reproductive abnormalities (epithelial hyperplasia, dysplasia, and apoptosis) in the transplanted uteri are evident only if both the donor and recipient were exposed to DES (Hendry et al., 1997). No lesions were seen in uteri from control animals transplanted into cheek pouches of neonatal DES treated 7-day-old Syrian hamster recipients. This suggests that prenatal treatment initiates lesions, which are promoted by a second DES exposure. Finally, the uterine tissues

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from neonatal DES treated mice that were subsequently given estrogen, had significantly higher percentages of estrogen receptor mRNA, c-fos and c-jun (Kamiya et al., 1996). These studies suggest that prenatal exposure to estrogenic compounds can lead to an "imprinting" on the immune system permanently altering the cellular response to hormone exposure during adulthood. In our previous study, we found that mice prenatally exposed to DES had an altered immune response to *in-vitro* splenocyte stimulation only when exposed to a second dose of DES later in life (Karpuzoglu-Sahin et al., 2001). In this study, we investigated how prenatal exposure to DES followed by a secondary exposure at one year of age effects the thymus and T-cell subsets and development of autoimmunity. We show that immunological responses to DES are gender specific.

Materials and Methods

Animal Breeding and Experimental Design

All mice used in this study were C57BL/6 strain obtained from Charles River Laboratories (Wilmington, MA). In order to optimize breeding, two female mice were housed with one male. All female body weights were recorded before breeding began. Vaginal plugs were checked once a day for 5 days. The female mice were moved to separate cages immediately after a vaginal plug was found. A successful breeding was noted by a significant increase in body weight one week after the vaginal plug was found. On day 14 of gestation, the mice were administered, via subcutaneous injection, 0.25µg or 2.5µg of DES (Sigma, St. Louis, MO) in 0.05mL autoclaved tocopherol stripped corn oil (ICN, Auro, OH). Mice that received autoclaved tocopherol-stripped corn oil only served as negative controls. Mice were

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fed a commercial pelleted diet devoid of synthetic estrogens (Special diet #7013

Harlan Teklad, Madison, WI) and given water *ad-libitum*. All animals were housed at

a 14-h light: 10-h dark cycle. When the mice exceeded one year of age, they received

one subcutaneous injection of 0.03 μg/g bw of DES dissolved in 0.05mL corn oil. The

rationale for the second dose of DES in adult life is based on the findings from our

recently published study, where we noticed that immunoregulatory abnormalities (e.g.

enhanced IFNγ secretion) in prenatal DES exposed mice become evident after the

second exposure to DES in adult life (Karupuzoglu-Sahin, 2001a). Prenatal DES

exposure appears to preprogram the highly sensitive fetal immune system to respond

aberrantly when the individual is exposed to a second dose of estrogenic endocrine

disrupter (e.g. DES). For the purpose of a separate study, six days after the mice

were given a second dose of DES, they were challenged with 20 μg of RH strain *T.*

gondii antigen dissolved in 10 μl of injectable sterile saline (0.9% sodium chloride

injection USP). One to two months later, the mice were anesthetized with halothane

and terminated via cervical dislocation. Thus, there were two treatment groups which

included: (1) prenatal DES/adult DES + *T. gondii* and (2) prenatal oil/ adult DES + *T.*

gondii. All procedures were in accordance with the guidelines of the animal care

committee of the Virginia Polytechnic Institute and State University. Groups of mice

were terminated on two separate days with equal number of mice from each treatment

group on both days. The experiment was controlled for error by using the generalized

randomized complete block design (Lenter, 1993).

Blood Collection

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All mice were anesthetized with halothane immediately before collecting blood. Blood

was collected through the retroorbital sinus puncture using heparin coated glass

micro-capillary tubes at the time of termination.

Isolation of Thymus and Thymic Lymphocytes

The thymus was aseptically isolated, trimmed of all excess body fat and gently blotted

on sterile gauzes to remove excess blood. Each thymus was weighed and then

dissociated by gently grinding the organ on a sterile steel wire mesh (Sigma, Saint

Louis, MO) in phenol-red depleted RPMI-1640 incomplete media (Mediatech,

Herndon, VA) (Calemine et al., 2002). Cell suspensions were centrifuged at 200xg for

5-8 minutes at 4^0C and resuspended in complete media. The complete media

consisted of 10% heat-inactivated fetal bovine serum (FBS) that was pretreated with

charcoal and dextran (Atlanta Biologicals, Atlanta, GA) to eliminate any estrogenic

compounds in the serum. Care was taken to use media that is devoid of phenol red,

which has been shown to have estrogenic effects. The media also contained 200mM

L-glutamine, 5000 IU/mL penicillin, 5000 μg/mL streptomycin, and 100 x non-essential

amino acids. Cell numbers and cell size analyses were determined by Casy-1 Cell

Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany) and

adjusted to 5 x 10^6 cells/mL as described in our previous publications (Donner et al.,

1999).

Phenotyping of Lymphocytes by Flow Cytometric Analysis

One hundred microliters of freshly isolated thymocytes at a concentration of 5 X 10^6 cells/mL

(5 X 10^5 cells/well) were plated in Corning Cell Wells™ 96-well round-bottom tissue-culture

plates (Corning, Corning, NY).   Cells were washed in PBS to avoid any inadvertent

stimulation from the FBS- supplemented media. Aliquots of splenocytes were dual color

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stained immediately after harvesting with several different combinations of fluorescein

isothiocyanate (FITC) and phycoerythrin (PE) conjugated anti-mouse antibodies

(Pharmingen, San Diego, CA).   Aliquots of thymocytes were dual color stained immediately

after harvesting with either (1) anti-CD4-FITC and anti-CD25-PE or (2) anti-CD4-FITC, anti-

CD8-FITC and anti-CDVβ8TCR-PE. Thymocytes were also stained with the relevant

isotype-matched FITC and PE anti-rat $IgG_{2a}\kappa$ control antibodies and subjected to flow

cytometric analysis by procedures reported in our earlier studies (Donner et al, 1999,

Verthelyi et al, 1998). The data were analyzed with the Immuno-4 software program (Donner

et al., 1999, Ahmed and Sriranganathan, 1994).

Culturing of Thymocytes with Dexamethasone

One hundred micoliters of thymocytes, at a concentration of 5 x 10^6 cells/mL (5 x 10^5

cells/well), were plated in Corning 96-well round bottom tissue culture plates (Corning, NY).

One hundred microliters of DEX (1μg/mL) (Sigma, St. Louis, MO) in complete medium were

added to culture wells to bring the final volume to 200 μl. Control cultures consisted of

adding 100 μl complete. Cells were harvested 24 hours after culture for apoptosis analysis.

7-AAD Staining of Lymphocytes

Thymocytes were triple stained with FITC- anti-CD8 antibodies and PE-anti-CD4 antibodies

and 7-aminoactinomycin D (7-AAD) immediately after harvest and 24 hours after culturing in

dexamethasone (DEX).   As described in our previous studies (Calemine et al, 2002, Gogal

et al, 2000), one hundred microliters of cells at a concentration of 5 X 10^6 cells/mL were

plated in Corning Cell Wells™ 96-well round-bottom tissue-culture plate (Corning, Corning,

NY). Cells were dual stained with anti-CD4 and anti-CD8 monoclonal antibody cocktail for

30 minutes as mentioned above. Cells were then washed in cold PBS. Following the wash,

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100 μl of the DNA binding dye, 7-AAD (10 μg/ml; Molecular Probes, Eugene, OR) in a

supplemented buffer (0.1% BSA, 0.1% NaN_3, 1.0% fetal bovine serum in PBS) were added

to each well. The cells were incubated at 4^0 C for 20 minutes and then immediately analyzed

by the flow cytometer. Each subset of T-cells ($CD4^-8^-$, $CD4^+8^+$, $CD4^+8^-$, $CD4^-8^+$) was gated

and the percentages of live and apoptotic cells were determined by 7-AAD analyses.

Staining of cells with 7-AAD allows the identification of cells at three stages of cell

viability/apoptosis, these include: 7-AAD^{dull} (live), $7\text{-AAD}^{intermediate}$ (early apoptosis), and 7-

AAD^{bright} (late apoptosis/necrotic) (Donner et al, 1999, Gogal et al, 2000). In our previous

studies, we have shown that 7-AAD analyses closely match with FITC-annexinV and are

superior to propidium iodide and forward/side scatter analysis (Donner et al, 1999).

Statistics

An ANOVA was performed to test for main effects of dose and gender as well as their

interaction. Post-hoc mean separation was conducted using multiple comparisons. The

MIXED procedure of the SAS System (ver. 8.1, SAS Institute Inc., Cary, NC 27513) was

used to perform the calculations. P-values less than 0.05 were considered significant. Data

are represented as mean ± standard error of the mean (SEM).

Results

Effects of prenatal exposure to DES on the thymus, a primary lymphoid organ:

Sex differences in thymic weights were evident between female and male mice that

were prenatally given oil (**Figure 3.1A** and **B**). Female prenatal oil-treated mice had

increased thymic weights (especially when normalized to their body weights)

compared to age-matched prenatal oil-treated males (**Figure 3.1B**). Interestingly, the

response of thymus to prenatal DES exposure varied between sexes. In female mice,

prenatal DES exposure had no discernable effects on thymic weights (**Figure 3.1**). In

contrast, prenatal DES exposed male mice, unexpectedly, had increased the thymic

weights compared to gender-matched oil-treated controls (**Figure 3.1**). Importantly,

although female prenatal DES-exposure had no noticeable effects on thymic weights,

the total number of thymocytes was markedly decreased compared to gender-

matched controls (**Figure 3.2A**). **Figure 3.2B** shows a representative profile of cell

size analysis in prenatal oil or DES exposed female and male mice. As is evident in

Figure 3.2B, a marked diminution in thymocytes (between 5 and 15 μM) is evident in

female prenatal DES-exposed mice compared to gender-matched controls. In

contrast to female prenatal DES-exposed mice, in males that were prenatally exposed

to DES, there was no decrease in thymocyte numbers; rather there was a biological

trend of increase in thymocyte numbers (**Figures 3.2A** and **B**). The thymocyte

number data in male prenatal DES-exposed mice is consistent with the increased

thymic weights noticed in Figure 1A and B.

Effects of prenatal exposure to DES on phenotype of thymocyte subsets and

changes in apoptosis:

Since prenatal DES-exposure altered the numbers of thymocytes in a gender-related

fashion, a logical next step was to determine whether changes in thymocyte numbers

were a reflection of alterations in selected subsets of thymocytes. Based on the

presence or absence of CD4 and/or CD8, thymocytes can be broadly categorized as

immature ($CD4^-CD8^-$, $CD4^+CD8^+$) and mature thymocyte subsets ($CD4^+CD8^-$ and

$CD4^-CD8^+$) (**Table 3.1A**). There were no noticeable alterations in any of the

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thymocyte subsets after prenatal exposure (**Table 3.1A**). However, since DES affected the number of thymocytes per thymus, it became relevant to normalize these subsets to the total number of thymocytes per thymus. In female prenatal DES-exposed mice, there was a significant decrease in immature $CD4^-CD8^-$ and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocyte subsets per thymus compared to prenatal oil-treated female counterparts (**Table 3.1B**). There was also a biological trend of decrease in $CD4^+CD8^+$ in female prenatal DES-exposed mice (although it did not approach the statistical significance level) (**Table 3.1B**).   There was a significant decrease in the number of $CD4^-CD8^+$, $CD4^-CD8^-$, $CD4^+CD8^+$ in female prenatal DES exposed mice compared to prenatal DES-exposed male counterparts.

To investigate whether prenatal DES-treatment affected apoptosis in selected thymocyte subsets, each thymocyte subset was gated and analyzed for apoptosis based on the staining with 7-AAD. **Table 3.2** shows the relative percentages of early apoptosis, late apoptosis/necrosis and total apoptosis in freshly isolated thymocytes from prenatal DES or oil-treated mice. Sex differences in percent of apoptosis were evident in $CD4^+CD8^-$ and $CD4^-CD8^+$ subsets in prenatal oil-treated male and female mice.   Cells from males had significantly more live (and conversely less apoptotic) cells compared to females. However, prenatal DES-treatment did not reveal any significant differences (with the exception of increased apoptosis of $CD4^-CD8^+$ cells in males prenatally-treated with DES).

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Response of thymocytes from prenatal DES exposed mice to apoptosis inducing agent, Dexamethasone

Thymocytes from prenatal DES or oil-treated mice were exposed *in vitro* to dexamethasone, an apoptosis-inducing compound for a short term. This was done to ascertain: (1) whether prenatal DES-exposure alters the response of thymocytes to dexamethasone, and (2) whether sex-differential responses occur. **Tables 3.3** and **3.4** show the relative percentages of apoptotic cells in four major subsets of thymocytes (from oil and DES-treated female and male mice) after *in vitro* exposure to dexamethasone. An interesting pattern emerged from these cultures. There were marked differences in percentages of apoptotic CD4$^+$CD8$^+$ cells from female oil and DES-prenatal treated mice that were cultured in the absence of dexamethasone (media only). CD4$^+$CD8$^+$ from female oil-treated mice were significantly more viable (i.e. less apoptotic) than comparable cultures from female DES-treated mice. Interestingly, the incidence level of apoptosis of CD4$^+$CD8$^+$ thymocytes from female prenatal DES-treated mice that were cultured in the *absence of dexamethasone* (i.e. media only) was comparable to the dexamethasone-exposed CD4$^+$CD8$^+$ thymocytes from female oil controls (**Table 3.3** and **Figure 3.3**). Moreover, further apoptosis of this CD4$^+$CD8$^+$ subset from female prenatal DES-exposed mice could not be enhanced by the deliberate addition of dexamethasone, unlike that noticed in comparable cultures from oil-treated female controls. This selective increase in spontaneous apoptosis of CD4$^+$CD8$^+$ thymocytes in prenatal DES-treated mice was evident only in female mice, but not their male counterparts (**Table 3.3** and **3.4**, and **Figure 3.3**). Male CD4$^+$CD8$^+$ thymocytes from prenatal DES-exposed mice were as

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sensitive to dexamethasone as oil controls.

Effects of prenatal exposure to DES on forbidden autoreactive cells and regulatory cells

Next, we examined whether prenatal DES exposure altered the expression of autoreactive ($V\beta 8^+$) or regulatory ($CD4^+CD25^+$) cells. The rationale for these studies was based on the fact that estrogenic compounds have been linked to autoimmune diseases (Ahmed 2000). Further, DES exposure during the critical fetal period may have altered thymic developmental pathways leading to the expression of autoreactive cells or changes in regulatory cells (that are believed to be important in down-regulating autoimmunity). Our findings show that prenatal DES-treatment did not significantly alter the *relative* percentages of autoreactive $V\beta 8^+$ thymocytes or $CD4^+CD25^+$ regulatory cells (**Table 3.5A**). However, total $V\beta 8^+$ x 10^6 cells / thymus was decreased in female prenatal DES-exposed mice compared to gender matched controls (**Table 3.5B**). In males, there was an increase in $CD4^+CD25^+$ x 10^6 regulatory cells / thymus in prenatal DES-exposed mice compared to gender matched controls (**Table 3.5B**).

Discussion

The *in-utero* environment can have many influences on fetal development. Exposure to synthetic estrogenic compounds such as DES during pregnancy has been associated with the development of immune dysfunction, high incidence of reproductive organ-specific cancers, and autoimmune diseases. However, there are no current immunological studies which explore the consequences of prenatal

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exposure to DES in both genders. This study was designed to investigate the immunological effects of prenatal exposure to DES on a cellular level in both females and males.

We found that *in-utero* exposure to DES can cause long-lasting gender specific immunological effects on the thymus. First, we reported DES effects on the thymic weight and cellularity. DES exposure, which has been previously shown to target the thymus and induce atrophy, was seen to have the opposite effect in male mice in this study. DES prenatally exposed male mice had an increase in thymic weight and a biological increasing trend in thymocyte count. We expected with such an appreciable increase in thymic weight and thymic weight to body weight ratio that there would have been a comparable increase in thymocyte numbers. Since this was not the case, one possibility to consider is that DES is targeting an increase in reticuloendothelium cells, nurse cells and/or vascular tissues of the thymus. Although the exact mechanism is unknown, prenatal exposure to estrogenic compounds has been noted to increase thymic weight in males. A recent study on the effects of prenatal exposure to phytoestrogen, genistein, on the endocrine and immune systems showed thymic masses were greater in treated male rats compared to males exposed to no genistein (Klein et al., 2002).

Females, on the other hand, had no significant changes in thymic weight, yet had a significant decrease in thymocyte count which has been reported in studies with DES exposed mice (Hirahara et al., 1994, Forsberg, 1984). We took a closer look into which subset of thymocytes were being targeted by prenatal DES treatment. We

found that the relative percentages of all four T-cell subsets were comparable in both

genders given oil or DES prenatal treatment, yet the total numbers of each thymocyte

subset were lower in DES treated female mice compared to oil treated controls.

Prenatal exposure induces a decrease in thymocyte populations over all subsets.

There are four possibilities as to how DES induces thymic atrophy: (1) damage to

thymic epithelium, (2) damage to stem cell populations, (3) lack of prothymocyte

migration which could be a result of decrease in Notch-1 surface markers and/or (4)

direct thymocyte apoptosis. *In-utero* exposure to DES has been shown to decrease

the number of prothymocytes in mouse fetal liver (Holladay et al., 1993). We

investigated the possibility that prenatal DES exposure may induce apoptosis of adult

thymocytes. Although there was no apparent increase in apoptotic T-cells analyzed

immediately after harvest, abnormal patterns of apoptosis were noticed in thymocytes

from DES treated mice after culturing for 24 hours in the absence of any known

stimulants. Thymocytes from DES treated female mice had a significant increase in

total apoptotic $CD4^+CD8^+$ cells after culturing in media for 24 hours compared to

thymocytes from oil treated controls. In addition, the level of apoptosis noticed in

media cultured thymocytes from DES treated mice was comparable to the DEX

cultured thymocytes from oil treated female mice. The above observations suggest

that DES exposure causes increased apoptosis of the $CD4^+CD8^+$ cells. This implies

that prenatal exposure to DES resulted in an intrinsic defect in the thymus, particularly

in $CD4^+CD8^+$ cells, that was long-lasting in females only. Similar findings have been

reported. For instance, DES and genistein induced thymic atrophy, decreased

cellularity, and induced apoptosis of CD4+CD8+ cells have been reported in several

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adult female animal studies (Okasha et al., 2001, Yellayi et al., 2002, Gould et al., 2000).

A T-cell subpopulation, $CD4^+CD25^+$, has been shown in several publications to prevent the development of T-cell mediated autoimmune diseases such as gastritis and thyroiditis. In fact, studies have shown that when $CD4^+CD25^+$ cells are removed from normal mice, autoimmune diseases develop without any exogenous self-antigen stimulations. However, when mice were reconstituted with $CD4^+CD25^+$ cells, they were able to recover from the disease (Sakaguchi, 1985, Smith, 1992, Suri-Payer et al., 1998). $CD4^+CD25^+$ T-cells make up approximately 5% of the total thymocyte population and 10% in the periphery (Shevach, 2002, Itoh et al., 1998).   In this study, we found that DES exposure did not have a significant effect on the relative percentages of thymic $CD4^+CD25^+$ population. Since these mice were one-year of age, it is possible to expect a natural depletion of thymocytes with age including the depletion of $CD4^+CD25^+$ subpopulation. The average relative percentage of $CD4^+CD25^+$ cells recovered in this study was only 1.4%, which is significantly lower than the expected 5% previously reported. Therefore, $CD4^+CD25^+$ cells may have been able to escape any influences of the DES exposure because there were few cells present. However, we did notice a significant increase in the number of $CD4^+C25^+$ cells in DES treated male mice when normalized to the total number of thymocytes. This increase is reflective of the non-significant increasing trend in total thymocyte count in males treated with DES. Still, studies have shown mice given only

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a few CD4$^+$CD25$^+$ cells had protection against developing autoimmunity such as EAE (experimental allergic encephalomyelitis). Therefore, the increase in the number of CD4$^+$CD25$^+$ cells in male mice given DES may have beneficial effects. Still, more studies are warranted this area.

We also evaluated the numbers of the autoimmune associated T-cell subset, TCRVβ8$^+$, in the thymus of female and male mice exposed to DES prenatally. There was no appreciable difference between the relative percentages of TCRVβ8$^+$ cells in either gender given oil or DES treatment. This is most likely because the relative percentages were too low to have a measurable effect.   However, the total number of autoreactive T-cells decreases along with all other T-cell populations in the thymus of DES treated female mice. These data suggest female mice given DES have less autoreactive T-cells than their gender matched oil treated controls. Still, more studies involving prenatal DES exposure in autoimmune prone mice are needed to conclusively evaluate the role of DES exposure in the advancement or protection against autoimmunity.

The results of this study suggest that males and females have different immunological responses to prenatal exposure to DES. The thymus, a primary lymphoid organ, is responsible for the education and maturation of T-cells. Critical steps in the T-cell development, particularly those involved in the negative selection of self-reactive Tcells, are vital for a healthy immune system. In this study, we show that prenatal exposure to DES can cause a disruption in the development of thymic lymphocytes. Females exposed to DES prenatally had an overall decrease in thymocyte numbers

and an increase in the number of apoptotic immature T-cells. The above observations

may explain why DES daughters were prone to developing autoimmune diseases later

in life. These effects of DES were long lasting since significant differences from

prenatal exposure were seen in mice at one-year of age.   The data suggest that

prenatal exposure to DES may leave an early imprinting on the immune system, which

is unique to females.

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A
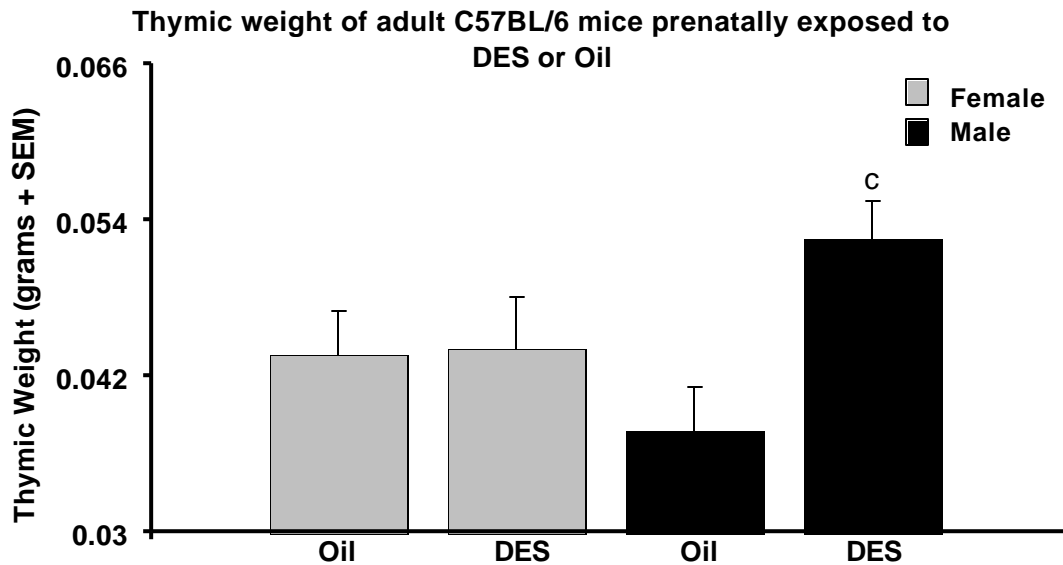
Thymic weight of adult C57BL/6 mice prenatally exposed to DES or Oil

c denotes significant difference between males given oil and males given DES

B

Thymic weight : body weight ratio of adult C57BL/6 mice prenatally exposed to DES or Oil
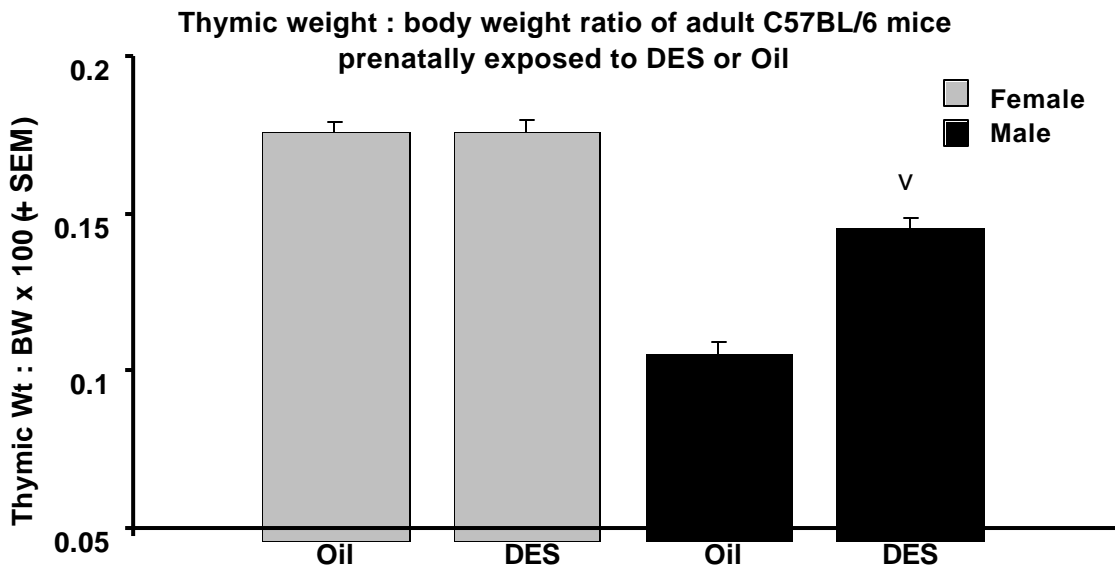
v denotes significant difference between males given oil and males given DES

Figure 3.1 Thymic weight and thymic body weight ratios of adult C57BL/6 mice prenatally exposed to DES or oil: C57BL/6 mice were exposed to either DES (0.25μg) (n_{female}= 7, n_{male}= 11) or oil (n_{female} = 10, n_{male}= 8) during prenatal development followed by a second exposure at 1yr old. The thymus was extracted from each mouse, weighed (Figure 1A) and related to total body weight (Figure 1B). The data are presented as mean ± standard error of the mean. Differences between genders and prenatal treatment were considered significant when $P<0.05$.

A. **Thymic cellularity of adult C57BL/6 mice prenatally exposed to DES or Oil**

☐ Female
■ Male

Thymocyte Count (x 10^6 cells + SEM)

60
50
40
30
20
10

a,b

Oil   DES   Oil   DES
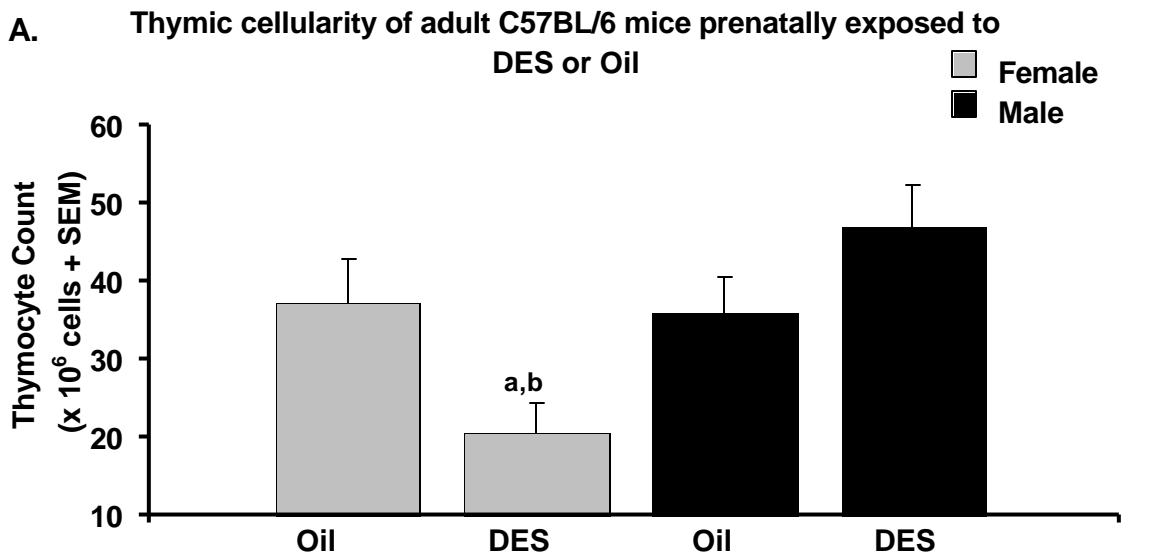
a denotes significant difference between females given oil and females given DES
b denotes significant difference between females given DES and males given DES

B.

Oil

DES

Female

41.8×10^6 cells/mL

26.9×10^6 cells/mL

100
80
60
40
20

2.5 5.0 7.5 10.0 12.5 15.0mm

100
80
60
40
20

2.5 5.0 7.5 10.0 12.5 15.0mm

Male

35.2×10^6 cells/mL

48.7×10^6 cells/mL

100
80
60
40
20

2.5 5.0 7.5 10.0 12.5 15.0mm

100
80
60
40
20

2.5 5.0 7.5 10.0 12.5 15.0mm
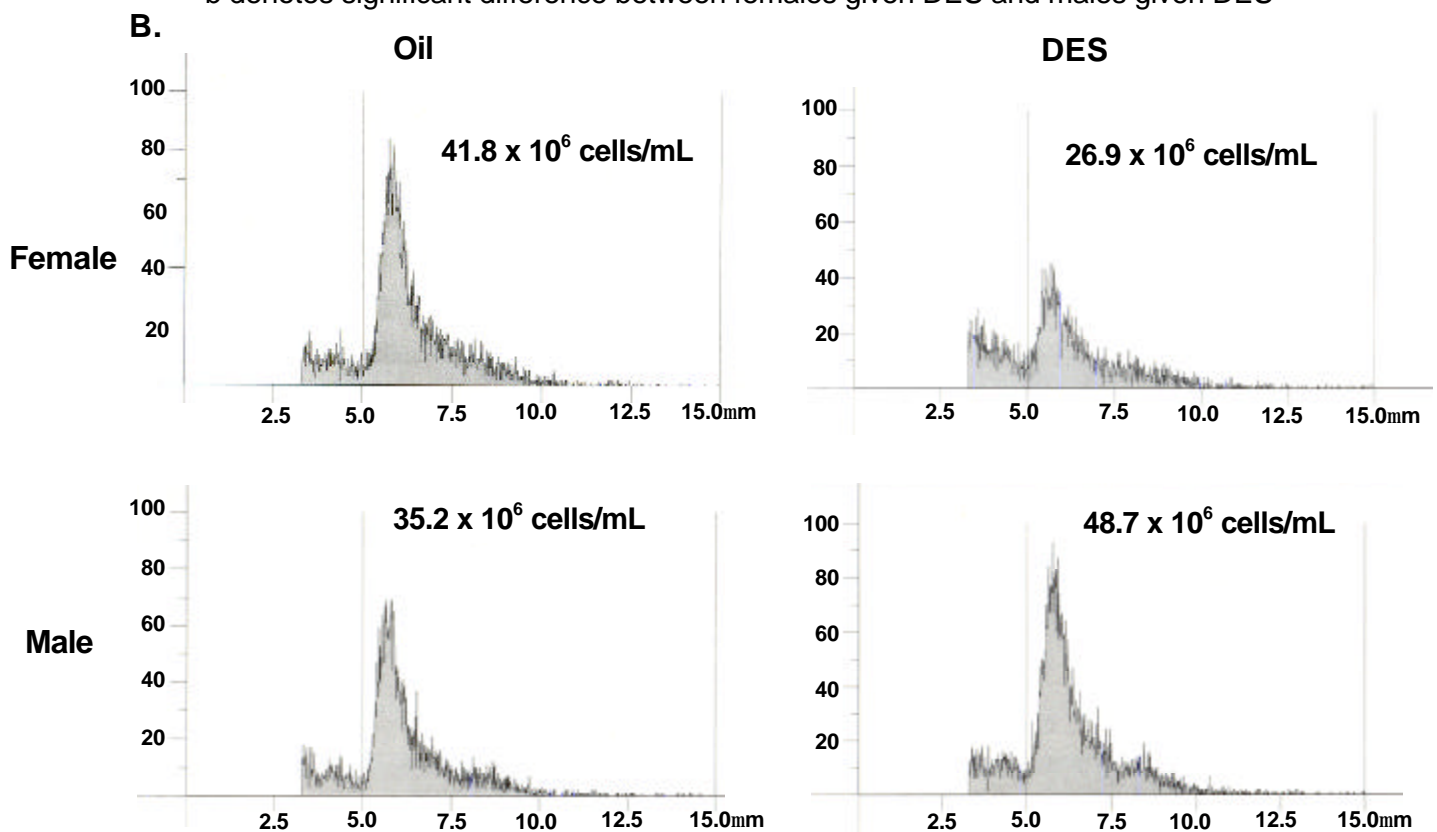
Figure 3.2 Thymic cellularity of adult C57BL/6 mice prenatally exposed to DES or oil: C57BL/6 mice were exposed to either DES (0.25µg) (n_{female}= 7, n_{male}= 11) or oil (n_{female} = 10, n_{male}= 8) during prenatal development. At one year of age all mice were given 30µg/kgbw DES. Immediately after harvesting the thymus, thymocyte suspensions were counted on a Casy-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany). Figure 2A data presented are mean ± standard error of the mean. Figure 2B representative Casy-1 data is shown in Figure 2b.
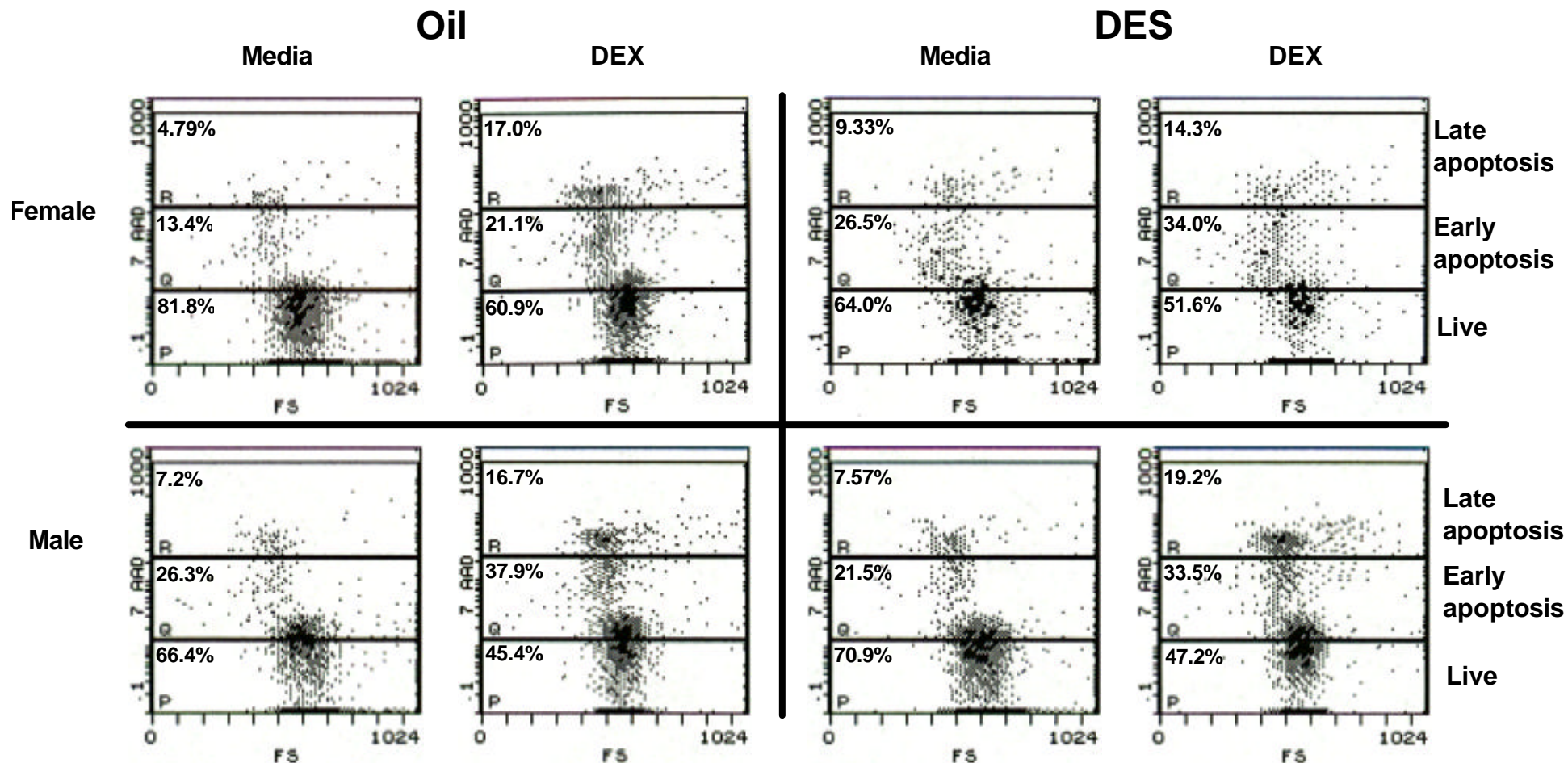
Figure 3.3 Triple color 7-AAD staining of cultured thymocytes from oil and DES prenatally treated female and male mice: C57BL/6 mice were exposed to either DES (0.25µg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development followed by a second exposure at 1yr old. Thymocytes were stained for T-cell subset markers with PE-anti-CD4 and FITC-anti-CD8 antibodies and 7AAD. Control cultures were stained with fluorochrome tagged PE and FITC-isotype matched antibodies and unstained (third color control for 7-AAD). Thymocyte subpopulations were gated and analyzed for 7-AAD staining. This DNA binding dye identifies three distinct subsets, 7-AAD[dull] or live, 7-AAD[intermediate] or early apoptotic and 7-AAD^{bright} late apoptotic. The data presented are CD4^{+}CD8^{+} thymocyte percentages from representative mice, one mouse per treatment group.

Table 3.1A Relative percentages of anti-CD4-PE, anti-CD8-FITC stained thymocytes from DES or oil prenatally exposed mice immediately after harvest.

Thymocyte Subset	Female		Male	
	Oil	DES	Oil	DES
CD4$^-$CD8$^-$	15.7 ± 4.0	9.9 ± 4.1	8.2 ± 4.0	7.0 ± 4.4
CD4$^+$CD8$^+$	68.6 ± 4.1	73.6 ± 4.4	74.7 ± 4.3	77.7 ± 4.2
CD4$^+$CD8$^-$	12.2 ± 1.7	11.2 ± 1.8	12.9 ± 1.4	10.5 ± 1.2
CD4$^-$CD8$^+$	4.0 ± 1.1	4.6 ± 1.3	4.2 ± 1.2	4.9 ± 1.3

Table 1B Thymocyte subsets x 10^6/thymus

| Thymocyte Subset	Female		Male	
	Oil	DES	Oil	DES
CD4$^-$CD8$^-$	3.1 ± 0.5	2.1 ± 0.4 [f,g]	2.9 ± 0.4	3.1 ± 0.4
CD4$^+$CD8$^+$	28.3 ± 7.9	17.0 ± 7.5 [g]	27.7 ± 8.0	38.5 ± 7.7
CD4$^+$CD8$^-$	4.4 ± 1.3	2.6 ± 1.2 [f]	4.9 ± 1.4	5.2 ± 1.4
CD4$^-$CD8$^+$	1.5 ± 0.6	0.9 ± 0.2 [f,g]	1.6 ± 0.2	2.0 ± 0.3

f denotes a significant difference between females given oil and females given DES
g denotes a significant difference between females given DES and males given DES

C57BL/6 mice were exposed to either DES (0.25μg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development. At one year of age all mice were given 30μg/kgbw DES. Thymocytes were stained for T-cell subset markers CD4 and CD8 immediately after harvest. The data are presented as mean ± standard error of the mean. Differences between genders and prenatal treatment were considered significant when *P*<0.05.

Table 3.2 Relative percentages of anti-CD4-PE, anti-CD8-FITC and 7-AAD stained thymocytes from DES or oil prenatally exposed mice immediately after harvest.

Thymocyte Subset		Female		Male	
		Oil	DES	Oil	DES
CD4⁻CD8⁻	Live cells	77.6 ± 9.4	75.8 ± 9.3	83.7 ± 9.4	81.6 ± 9.2
	Early apoptosis	18.9 ± 9.0	20.0 ± 8.9	14.7 ± 9.0	17.0 ± 8.8
	Late apoptosis	3.4 ± 0.5^a	3.4 ± 0.5	1.7 ± 0.5^a	2.0 ± 0.5
	Total apoptosis	22.4 ± 9.4	24.1 ± 9.3	16.3 ± 9.2	18.4 ± 9.2
CD4⁺CD8⁺	Live cells	83.4 ± 8.7	75.8 ± 8.4	89.6 ± 8.7	89.1 ± 8.4
	Early apoptosis	11.4 ± 2.9	14.8 ± 2.8^c	7.7 ± 2.9	7.0 ± 2.7^c
	Late apoptosis	4.9 ± 5.3	8.9 ± 5.2	2.7 ± 5.3	3.8 ± 5.1
	Total apoptosis	16.4 ± 7.5	23.9 ± 7.2^c	10.2 ± 7.5	10.8 ± 7.1^c
CD4⁺CD8⁻	Live cells	78.9 ± 2.8^a	82.1 ± 2.9	86.5 ± 2.9^a	85.3 ± 2.8
	Early apoptosis	12.6 ± 1.2^a	10.9 ± 1.4^c	8.2 ± 1.3^a	7.4 ± 1.3^c
	Late apoptosis	8.4 ± 2.2	7.2 ± 2.2	5.1 ± 2.3	7.7 ± 2.2
	Total apoptosis	21.1 ± 2.8^a	17.9 ± 2.9	13.5 ± 2.9^a	14.7 ± 2.8
CD4⁻CD8⁺	Live cells	77.8 ± 2.1^a	79.5 ± 2.1	83.6 ± 2.1^{ab}	77.9 ± 2.1^b
	Early apoptosis	16.6 ± 1.2^a	15.3 ± 1.2	13.4 ± 1.2^{ab}	17.7 ± 1.2^b
	Late apoptosis	5.7 ± 2.7	5.4 ± 2.7	2.3 ± 2.7	4.5 ± 2.6
	Total apoptosis	22.3 ± 2.1^a	20.5 ± 2.1	16.4 ± 2.1^{ab}	22.1 ± 2.1^b

[a] **significant difference between females given oil and males given oil**

[b] **significant difference between male mice given oil and male mice given DES**
[c] **significant difference between females given DES and males given DES**

C57BL/6 mice were exposed to either DES (0.25µg) ($n_{female}= 6$, $n_{male}= 6$) or oil ($n_{female} = 6$, $n_{male}= 6$) during prenatal development. At one year of age all mice were given 30µg/kgbw DES. Thymocytes were stained for T-cell subset markers CD4 and CD8 immediately after harvest. The data are presented as mean ± standard error of the mean. Differences between genders and prenatal treatment were considered significant when $P<0.05$.

Table 3.3 Relative percentages of anti-CD4-PE, anti-CD8-FITC and 7-AAD stained thymocytes after 24 hours incubation with media or dexamethasone (DEX) from adult female C57BL/6 mice prenatally exposed to oil or DES

		Female					
		Oil			DES		
Thymocyte Subset		Media	DEX	% loss	Media	DEX	% loss
CD4⁻CD8⁻ cells	Live	50.3 ± 9.7	37.8 ± 9.3	12.5 ± 4.6	51.5 ± 9.7	37.1 ± 8.1	15.2 ± 4.5
	Early apoptosis	46.6 ± 8.4	58.4 ± 8.4		45.5 ± 8.4	58.9 ± 7.3	
	Late apoptosis	3.1 ± 1.4	3.8 ± 1.3		3.0 ± 1.4	3.6 ± 1.3	
	Total apoptosis	49.7 ± 9.7	62.2 ± 9.3		48.5 ± 9.7	60.9 ± 8.1	
CD4⁺CD8⁺ cells	Live	75.0 ± 6.1[a]	59.5 ± 3.8	15.5 ± 5.0	58.4 ± 5.6[a]	49.9 ± 3.8	**6.7 ± 4.6***
	Early apoptosis	19.0 ± 4.5[a]	28.6 ± 1.7		31.0 ± 4.1[a]	36.9 ± 1.7	
	Late apoptosis	6.0 ± 1.6	11.9 ± 2.5		10.1 ± 1.6	12.9 ± 2.4	
	Total apoptosis	**25.0 ± 6.1[a]**	40.5 ± 3.8		**41.5 ± 5.6[a]**	50.0 ± 3.8	
CD4⁺CD8⁻ cells	Live	57.9 ± 2.9	43.0 ± 4.8	15.0 ± 3.6	51.6 ± 2.9	35.0 ± 4.4	16.9 ± 3.6
	Early apoptosis	30.8 ± 2.7	38.1 ± 3.3		32.6 ± 2.7	46.4 ± 3.2	
	Late apoptosis	11.3 ± 1.7	19.0 ± 2.3		15.0 ± 1.7	20.5 ± 2.3	
	Total apoptosis	42.1 ± 2.9	57.1 ± 4.8		48.3 ± 2.9	65.1 ± 4.4	
CD4⁻CD8⁺ cells	Live	62.9 ± 6.9	43.4 ± 7.6	19.9 ± 4.2	59.2 ± 6.9	37.0 ± 7.1	23.4 ± 4.2
	Early apoptosis	26.4 ± 4.7	39.0 ± 4.9		31.5 ± 4.7	45.1 ± 4.8	
	Late apoptosis	10.7 ± 2.5	17.6 ± 2.7		9.3 ± 2.5	18.6 ± 2.7	
	Total apoptosis	37.1 ± 7.0	56.7 ± 7.6		40.8 ± 7.0	63.0 ± 7.1	

*denotes a significant difference between females given DES and males given DES (see Table 4)

[a]denotes a significant difference between females given oil and females given DES

C57BL/6 mice were exposed to either DES (0.25µg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development followed by a second exposure at 1yr old. Thymocytes were stained for T-cell subset markers with 7AAD. Thymocyte subpopulations were identified and gated. Each gated subset was analyzed for staining intensity with 7-AAD against forward scatter. Percent loss was calculated from the difference of relative percentages of live cells cultured in DEX and media. The data are presented as mean ± standard error of the mean. Differences between genders and prenatal treatment were considered significant when $P<0.05$.

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Table 3.4 Relative percentages of anti-CD4-PE, anti-CD8-FITC and 7-AAD stained thymocytes after 24 hours incubation with media or dexamethasone (DEX) from adult male C57BL/6 mice prenatally exposed to oil or DES

		Male					
		Oil			DES		
Thymocyte Subset		Media	DEX	% loss	Media	DEX	% loss
CD4⁻CD8⁻	Live cells	50.0 ± 9.7	42.8 ± 9.3	10.5 ± 4.6	50.5 ± 9.7	35.5 ± 8.0	13.0 ± 4.4
	Early apoptosis	45.8 ± 8.4	53.2 ± 8.4		45.9 ± 8.4	60.5 ± 7.2	
	Late apoptosis	4.2 ± 1.4	4.4 ± 1.3		3.6 ± 1.4	4.1 ± 1.3	
	Total apoptosis	50.0 ± 9.7	64.5 ± 8.0		57.5 ± 3.7	49.5 ± 9.6	
CD4⁺CD8⁺	Live cells	69.3 ± 6.2	51.3 ± 3.8	18.5 ± 8.5	64.0 ± 5.5	42.5 ± 3.7	**22.8 ± 7.4***
	Early apoptosis	23.7 ± 4.5	34.4 ± 1.7		27.3 ± 4.0	38.4 ± 1.7	
	Late apoptosis	7.0 ± 1.6	14.2 ± 2.5		8.9 ± 1.5	18.8 ± 2.4	
	Total apoptosis	30.7 ± 6.2	48.7 ± 3.9		36.0 ± 5.5	57.5 ± 3.7	
CD4⁺CD8⁻	Live cells	55.5 ± 2.9	38.1 ± 4.8	17.5 ± 3.7	50.8 ± 2.9	29.5 ± 4.3	20.5 ± 3.5
	Early apoptosis	28.2 ± 2.7	40.9 ± 3.4		33.6 ± 2.6	45.5 ± 3.1	
	Late apoptosis	16.2 ± 1.7	21.1 ± 2.3		16.1 ± 1.7	22.6 ± 2.3	
	Total apoptosis	44.5 ± 2.9	62.0 ± 4.8		49.1 ± 2.9	70.5 ± 4.3	
CD4⁻CD8⁺	Live cells	63.9 ± 6.9	36.5 ± 7.6	29.4 ± 4.2	60.0 ± 6.9	29.2 ± 6.9	27.6 ± 4.2
	Early apoptosis	28.1 ± 4.7	44.9 ± 4.9		30.3 ± 4.7	48.6 ± 4.7	
	Late apoptosis	8.0 ± 2.5	19.7 ± 2.7		9.7 ± 2.5	20.2 ± 2.7	
	Total apoptosis	35.8 ± 7.0	63.5 ± 7.7		40.0 ± 7.0	70.8 ± 6.9	

*denotes significant difference between females given DES and males given DES (see Table 3.3)

C57BL/6 were exposed to either DES (0.25µg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development followed by a second exposure at 1yr old. Thymocytes were stained for T-cell subset markers with 7AAD. Thymocyte subpopulations were identified and gated. Each gated subset was analyzed for staining intensity with 7-AAD against forward scatter. Percent loss was calculated from the difference of relative percentages of live cells cultured in DEX and media. The data are presented as mean ± standard error of the mean. Differences between genders and prenatal treatment were considered significant when $P<0.05$.

Table 3.5A Relative percentages of thymocytes stained immediately after harvest from female and male mice exposed to either oil or DES during

Thymocyte Type	Female		Male	
	Oil	DES	Oil	DES
$V\beta8^+$	1.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
$CD4^+CD25^+$	0.8 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	1.3 ± 0.2

prenatal development.

Table 3.5B Thymocytes x 10^6/thymus

Thymocyte Type	Female		Male	
	Oil	DES	Oil	DES
$V\beta8^+$	0.56 ± 0.04	0.33 ± 0.07^p	0.48 ± 0.06	0.69 ± 0.13
$CD4^+CD25^+$	0.39 ± 0.14	0.27 ± 0.01	0.31 ± 0.09	0.88 ± 0.19^m

[p] denotes a significant difference between females given DES and females given oil

[m] denotes a significant difference between males given DES and males given oil

C57BL/6 mice were exposed to either DES (0.25µg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development. At one year of age all mice were given 30µg/kgbw DES. Thymocytes were stained for autoimmune T-cell subset $V\beta8^+$ and regulatory T-cells which express $CD4^+CD25^+$ immediately after harvest. The data are presented as mean ± standard error of the mean. Differences between prenatal treatment were considered significant when $P<0.05$.

Chapter 4
Prenatal Exposure to Diethylstilbestrol Has Long-Lasting Immunological Effects on the Spleen

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Abstract

An exciting new area of medical research is understanding how the *in-utero* environment affects fetal development as well as health later in life. Studies have shown a variety of substances to affect the fetal development. For instance, alcohol consumption during pregnancy is now known to cause abnormal neurological developments in the fetus. New studies now show malnourishment early in the pregnancy can be associated with the development of high blood pressure. The present study shows that prenatal exposure to small amounts of diethylstilbestrol, a synthetic estrogen, once widely given to pregnant women, followed by a second dose later in adulthood can have long-lasting effects on the immune system. In this study, DES prenatally exposed females produced higher amounts of interferon-γ (IFNγ) in response to an *in-vivo T. gondii* antigenic challenge than females exposed *in-utero* to an oil only control treatment. Females given DES prenatally also have increased expression of CD28 co-stimulation marker on T-cells and produce higher levels of IFNγ in response to an *in-vitro* Con-A stimulation. Males prenatally exposed to DES did not have altered IFNγ levels or expression of CD28 in the *in-vivo* or *in-vitro* assays. The effects of prenatal DES exposure on the development of autoimmunity in both males and females is explored in this study. These results demonstrate that DES treatment can cause an "immunological imprint" on the fetal immune system altering the immunological responses to certain stimuli. These immunologically induced effects from DES treatment differ between genders.

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Introduction

Diethylstilbestrol (DES), developed in the late 1930s, has been identified as the most potent synthetic estrogenic compound. Administration of DES dates back to 1940 when it was primarily given to pregnant women who were at risk of developing serious complications and threatened spontaneous abortions. It was originally thought that administration of DES would help stabilize the natural estrogen levels and alleviate any risk of complications. The premature establishment of DES, as a therapeutic drug, on the healthcare market excluded any dose efficacy studies or risk assessment tests from being performed. Without such concrete analysis, DES dose administration was at the discretion of each prescribing doctor. This was evident since there was a wide range of doses reported during all stages of pregnancy. DES related health problems began to be noticed in the late 1960s when young women exposed to DES *in-utero* were diagnosed with a rare carcinoma, clear cell vaginal adenocarcinoma. Decades of retrospective studies have shown adverse reproductive effects including infertility and a variety of carcinomas in both men and women exposed to DES during prenatal development (Stillman, 1982, Sandberg et al., 1981, Burke et al., 2001, Golden et al., 1998). A recent questionnaire study of 4821 DES exposed daughters and 2095 unexposed women reported an excess risk of developing breast cancer at the age of 40 and over (Palmer et al., 2002).

Immunological related abnormalities have been frequently reported in DES

exposed mothers, daughters and sons including development of autoimmunity

(arthritis, asthma and lupus), increased susceptibility to respiratory and bladder

infections and an overall decrease in lymphocyte activation (Wingard and Turiel,

1988, Vingerhoets et al., 1998, Ablin et al., 1988, Ways et al., 1987, Burke et al.,

2001). Animals exposed to DES *in-utero* or during neonate development or later

in adult life have shown similar DES-induced immune alterations. DES treatment

in mice has led to reduced proliferative response to T-cell mitogens (Kalland et

al., 1979), decreased natural killer cell functions (Kalland et al., 1984), impaired

immunity against infections (Kittas and Henry, 1980, Luebke et al., 1984,

Fugmann et al., 1983) and induced autoantibodies to cardiolipin (Forsberg,

2000).

Currently, there is a strong interest in understanding the immunological

consequences of exposure to potent estrogenic compounds during fetal

development.   A recent study from our laboratory reported mice exposed to DES

during prenatal development had altered immune responses to *in-vitro*

stimulation only if they were exposed to a second dose of DES later in life. This

study supports these findings and focuses on the immune responses in both

genders. We found that DES exposure has gender-specific immunological

effects particularly when DES exposed mice were challenged *in-vivo* with *T.*

gondii antigens and *in-vitro* with Con-A stimulation. This study explores the long-

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lasting effects of DES on immunological responses to infection and development of autoimmunity.

Materials and Methods

Animal Breeding and Experimental Design

All mice used in this study were C57BL/6 strain obtained from Charles River Laboratories (Wilmington, MA). In order to have efficient breeding, two female mice were housed with one male mouse. All female body weights were recorded before breeding began. Vaginal plugs were checked once a day for 5 days. The female mice were moved to separate cages immediately after a vaginal plug was found. A successful breeding was noted by a significant increase in body weight one week after the vaginal plug was found. On day 14 of gestation, the mice were given via subcutaneous injection 0.25μg or 2.5μg of DES (Sigma, St. Louis, MO) in 0.05mL autoclaved tocopherol stripped corn oil (ICN, Auro, OH) or corn oil only (served as a negative control). The surviving offspring were fed a commercial pelleted diet devoid of synthetic estrogens (Special diet #7013 Harlan Teklad, Madison, WI) and given water *ad-libitum*. All animals were housed at a 14-h light: 10-h dark cycle. When the mice reached over one year of age, they were given one subcutaneous injection of 30μg/kg bw of DES dissolved in 0.05mL corn oil. Six days later the mice were challenged with 20μg of RH strain *T. gondii* dissolved in 10μl of injectable sterile saline (0.9% sodium chloride injection USP). The *T. gondii* was given via intraperitoneal injection. At the closing of the study, 1-2 months after *T. gondii* challenge, the mice were

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asphyxiated with halothane and terminated via cervical dislocation. All

procedures were in accordance with the guidelines of the animal care committee

of the Virginia Polytechnic Institute and State University. Thus, there were two

treatment groups which included: (1) prenatal DES/adult DES + *T. gondii* and (2)

prenatal oil/ adult DES + *T. gondii*. This experiment was performed on two

separate days with equal number of mice from each treatment group on both

days. The experiment was controlled for error by using the generalized

randomized complete block design (Lenter, 1993).

Preparation of Soluble *T. gondii*

Tachyzoites of the RH strain of *T. gondii* were maintained by serial passage in

bovine monocyte (BM) cells (a gift from John Dame, University of Florida). Host

BM cells were grown to monolayers in 75-cm^2 plastic cell culture flasks in RPMI

medium containing 10% (v/v) fetal bovine serum, 100 Units of penicillin G per ml,

100 μg of dihydrostreptomycin (GIBCO, Grand Island, New York) per ml. Cell

cultures were incubated at 37°C in a 95% air-5% CO_2 atmosphere.

Tachyzoites were collected for antigen preparation by first removing the

cell culture medium and replacing it with calcium and magnesium free Hanks

balanced salt solution (HBSS). The BM cells were then scrapped off the plastic

growth surface of the 75 cm^2 flask into the HBSS, and the suspension forced

through a 27-gauge needle to rupture most BM cells. The free tachyzoites were

separated from intact cells and most cellular debris by filtration through a 3-mm-

pore-size polycarbonate filter (Nuclepore Corporation, Pleasanton, California).

The numbers of tachyzoites present were determined by counting in a

hemacytometer. The organisms were suspended in injectable saline and frozen at -95^0 C for 3-5 days to insure no viability. The whole organisms were sonicated on ice for six 30-second pulses to make soluble T. gondii antigen. The protein concentration was quantified using a Bradford Protein assay. Mice were given an intraperitoneal injection of 20μg of the whole R.H. T. gondii organism.

Blood Collection

All mice were anesthetized with halothane immediately before collecting blood. Blood was collected via the retro orbital sinus cavity using heparin coated glass micro-capillary tubes six hours following the *T. gondii* challenge.

Analysis of Reproductive and Lymphoid Organs

Reproductive organs (uterus and ovaries from females, seminal vesicles and testes from males) and lymphoid organs (thymus and spleen) were isolated, trimmed of all excess body fat and blotted dry of any excess blood using sterile gauzes. The tissues were weighed and fixed in formalin for histopathological analysis as described in our previous publication (Calemine et al., 2002).

Analysis of Salivary Glands, Thyroid and Kidneys for lymphocyte infiltration

The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micron sections were cut and stained with H&E.

Isolation of Lymphocytes

Lymphocytes from thymus and spleen were aseptically dissociated by gently grinding these organs on a sterile steel wire mesh (Sigma, Saint Louis, MO) in phenol-red depleted RPMI-1640 incomplete media (Mediatech, Herndon, VA) by

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procedures previously reported (Calemine et al., 2002, Karpuzoglu-Sahin et al., 2001). Tris-ammonium chloride lysis buffer (pH 7.2) was added to the splenocyte suspension to remove erythrocytes before culturing. The cell suspensions were washed twice with RPMI-1640 at 200xg for 5-8 minutes at 4^0C and resuspended in complete media. The complete media consisted of 10% heat-inactivated fetal bovine serum that was pretreated with charcoal and dextran (Atlanta Biologicals, Atlanta, GA) to eliminate any estrogenic compounds in the serum. The media also contained 200mM L-glutamine, 5000 IU/mL penicillin, 5000 μg/mL streptomycin, and 100 x non-essential amino acids. Cell numbers were assessed by Casy-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany) and adjusted to 5 x 10^6 cells/mL.

Phenotyping of Lymphocytes by Flow Cytometric Analysis

One hundred microliters of freshly isolated splenocytes or thymocytes at a concentration of 5 X 10^6 cells/mL (5 X 10^5 cells/well) were plated in Corning Cell Wells™ 96-well round-bottom tissue-culture plates (Corning, Corning, NY). Cells were washed in PBS to avoid any inadvertent stimulation from the FBS- supplemented media. Aliquots of splenocytes were dual color stained immediately after harvesting and 24 hours after culture with ConA with several different combinations of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated anti-mouse antibodies (Pharmingen, San Diego, CA). Dual color staining combinations included (1) anti-CD4-FITC with anti-CD25-PE, (2) anti-CD19-FITC with anti-CD40L-PE, (3) anti-CD90-FITC with anti-CD28-PE, and (4) anti-CD4-FITC, anti-CD8-FITC and anti-CDVβ8-PE. Splenocytes were also stained with the relevant isotype-matched FITC and PE anti-rat

IgG$_{2a}$κ control antibodies and subjected to flow cytometric analysis by procedures

reported in our earlier studies (Calemine et al., 2002, Verthelyi et al., 1998). The data

were analyzed with the Immuno-4 software program (Donner et al., 1999, Ahmed et al.,

1994, Karpuzoglu-Sahin et al., 2001).

 7-AAD Staining of Lymphocytes

Splenocytes were triple stained with FITC- anti-CD8 antibodies and PE-anti-CD4

antibodies and 7-aminoactinomycin D (7-AAD) immediately after harvest and 24 hours

after culturing in Con-A and dexamethasone (DEX), respectively.   As described in our

previous studies (Gogal et al, 2000), one hundred microliters cells at a concentration of

5 X 10^6 cells/mL were plated in Corning Cell Wells™ 96-well round-bottom tissue-culture

plate (Corning, Corning, NY).   Cells were dual stained with anti-CD4 and anti-CD8

monoclonal antibody cocktail for 30 minutes as mentioned above. Cells were then

washed in cold PBS. Following the wash, 100 µl of the DNA binding dye, 7-AAD (10

µg/ml; Molecular Probes, Eugene, OR) in a supplemented buffer (0.1% BSA, 0.1%

NaN$_3$, 1.0% fetal bovine serum in PBS) were added to each well. The cells were

incubated at 4^0 C for 20 minutes and then immediately analyzed by the flow cytometer.

Each subset of T-cells (CD4$^-$8$^-$, CD4$^+$8$^+$, CD4$^+$8$^-$, CD4$^-$8$^+$) was gated and the

percentages of live and apoptotic cells were determined by 7-AAD analyses. Staining

of cells with 7-AAD allows the identification of cells at three stages of cell

viability/apoptosis, these include: 7-AAD^{dull} (live), 7-AAD^{intermediate} (early apoptosis), and

7-AAD^{bright} (late apoptosis/necrotic) (Donner, et al., 1999). In our previous studies, we

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have shown that 7-AAD analyses closely match with FITC-annexinV and are superior to propidium iodide and forward/side scatter analysis.

Cytokine Analysis of Serum and Cell Culture Fluids

Sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect IFNγ protein levels in serum and cell culture fluids. ELISA procedures for serum samples involved coating 96 well Maxisorp high-binding immunoassay plates (Fisher Scientific, Sowanee, GA) with 4.0μg/mL of purified anti-IFNγ clone R4-6A2 antibody (Pharmingen) diluted in 1XPBS. The plates were coated at 4^0C overnight. The plates were washed twice with PBS + 0.0005% Tween 20 and incubated in blocking buffer (1XPBS + 1%BSA) at room temperature for two hours. Plates were blotted dry and serum samples were added to wells at 1:2 dilution. The plates were covered and incubated at 4^0C for 18-22 hours. After 5 washes the plates were incubated with 0.5μg/mL of biotin conjugated anti-IFNγ antibody (Pharmingen). After six washes, the plates were incubated with horseradish peroxidase solution (Vector Labs, Burlingame, CA) for 30 minutes at room temperature. After eight washes, the plates were developed with TMB substrate (KPL, Gaithersburg, MD). The plates were read at 450nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). An ELISA procedure for cell culture fluids followed a similar procedure as above and has been previously described in Karpuzoglu-Sahin et al., 2001. The IFNγ protein levels were extrapolated using the linear region of the standard curve calculated by the SOFTMAX PRO, Molecular Devices Inc. Software.

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Statistics

An ANOVA was performed to test for main effects of dose and gender as well as their interaction. Post-hoc mean separation was conducted using multiple comparisons. The MIXED procedure of the SAS System (ver. 8.1, SAS Institute Inc., Cary, NC 27513) was used to perform the calculations. P-values less than 0.05 were considered significant. Data are represented as mean ± standard error of the mean (SEM).

Results

I. Animal Dosing and Survival of Offspring:

A total of fifteen female and 7 male mice were bred for one week. Only seven female mice became pregnant. Out of 7 total pregnant mice, two mothers were given corn oil (negative control), two mothers were given one dose of 2.5μg DES, and 3 mothers were given one dose of 0.25μg DES on day 14 of gestation. Low doses of DES were administered to simulate the realistic exposure levels of environmental estrogens.

Surviving liters included:

Liter #1: seven pups (4 females, 3 males) exposed to 2.5μg DES on day 14 of gestation
Liter #2: three pups (3 males) exposed to 0.25μg DES on day 14 of gestation
Liter #3: eight pups (3 females, 5 males) exposed to 0.25μg DES on day 14 of gestation
Liter #4: seven pups (4 females, 3 males) exposed to 0.25μg DES on day 14 of gestation
Liter #5: nine pups (5 females, 4 males) exposed to corn oil (control) on day 14 of gestation
Liter #6: nine pups (5 females, 4 males) exposed to corn oil (control) on day 14 of gestation

Overall the mortality of the offspring included:
Pregnant mice given oil treatment: No mortality
Pregnant mice given 0.25μg DES: 33% mortality

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Pregnant mice given 2.5μg DES: 46% mortality

It was not uncommon to notice that the prenatal DES exposed mothers neglected their offspring, thereby; the offspring were deprived of vital nutrition during this critical period. There were no physical abnormalities noticed in the offspring other than slow gain of body weight.

The following data are a comparison of prenatal exposure to DES (0.25μg) and oil (negative control) treatments. The one liter (Liter #1) that was exposed to 2.5μg of DES *in-utero* was not included in the data analysis because of the small sample population.

II. DES-induced immunological changes were investigated at the peripheral blood level as well as the spleen (a secondary lymphoid organ).

> A. Reproductive organs of both female and male mice were analyzed for effects of DES exposure. The organ weight was analyzed. The uterus was also analyzed for DES effects on epithelial cell height (the uterotropic assay) since DES has been shown to induce histological changes in the uterus.
>
> B. Blood was collected six hours after *T. gondii* challenge. The serum was analyzed for interferon-gamma (IFNγ) levels.
>
> C. The secondary lymphoid organ (spleen) was analyzed for several immunological endpoints including: (1) weight, (2) lymphocyte cell count, (3) cytometric analysis of T-cell and B-cell lymphocytes, autoreactive T-cells and B-cells and regulatory T cells. Splenocytes were cultured in Con-A or media only. Cultured splenic lymphocytes

were collected for cytometric analysis of T cells and B cells as well as

the expression of co-stimulatory marker CD28, regulatory cells and

autoreactive T-cells.   The splenocyte culture fluids were analyzed for

IFNγ.

Effects of prenatal exposure to DES on the reproductive organs and body weight:

Since the focus of this study was to investigate the immunological effects of DES,

a potent synthetic estrogenic compound, it is also important to see the effects of

DES on the reproductive system. **Table 4.1** shows the body weight, reproductive

organ weight and body weight to organ weight ratio. The body weights were not

changed due to the treatment. There was no significant difference in reproductive

organ weights between oil or DES treated mice in either males or females. The

uterus was processed for histological analysis of uterine epithelial cell height

(which is known to increase in DES treated mice). In this experimental design,

where we are testing the effects of prenatal exposure to DES later in life, we did

not see any significant changes in uterine epithelial cell height due to DES

treatment (data not shown).

Effects of prenatal exposure to DES on the immunological response to an in-vivo antigenic challenge

We had previously reported that splenocytes from mice exposed to a similar DES

dose regimen had an increase in IFNγ producing cells and IFNγ secretion when

cultured with a T-cell specific stimulator, ConA (Karpuzoglu-Sahin et al., 2001a).

In this study, we wanted to investigate whether prenatal exposure to DES would

alter the immune response to an *in-vivo* immune stimulant such as *T. gondii*. Six

hours after mice were given 20μg of *T. gondii* antigens, IFNγ levels were

measured in the serum. Female mice exposed to DES during prenatal

development had significantly higher levels of IFNγ in their serum compared to

the oil treated control female mice (**Figure 4.1**). DES treated male mice, on the

other hand, had a non-significant decrease in IFNγ levels compared to their

gender matched oil treated control.

Effects of prenatal exposure to DES on splenic lymphocytes responses to

in-vitro stimulation

In support of our previous study, *in-vitro* stimulation of splenocytes with Con-A

induced significant effects in IFNγ production particularly in female mice given

DES treatment. **Figure 4.2** shows the significant increase in IFNγ protein

secreted by Con-A stimulated female splenocytes whereas splenocytes from

male DES or oil treated mice had little to no change in IFNγ secretion. Con-A

stimulated male splenocytes had no significant alterations in IFNγ production.

These DES induced immune alterations could be due to altered lymphocyte

numbers.

Effects of prenatal exposure to DES on the splenic weight and cellularity

Changes in organ weight can be reflective of changes in organ cellularity. The

splenic weight was not significantly altered by DES treatment in either females or

males (Female$_{oil}$ 0.09 $\pm$ 0.004g, Female$_{DES}$ 0.08 $\pm$ 0.005g, Male$_{oil}$ 0.07 $\pm$ 0.004g,

Male$_{DES}$ 0.08 $\pm$ 0.004g). The splenic weight to body weight ratio of female mice

given DES treatment during prenatal development had a decreasing trend compared to oil treated female control mice (Female$_{oil}$ 0.37 ± 0.01, Female$_{DES}$ 0.33 ± 0.02). Male splenic weight to body weight ratio of DES treated mice have an increasing trend compared to oil treated male control mice (Male$_{oil}$ 0.21 ± 0.01, Male$_{DES}$ 0.24 ± 0.01). Female splenic weight to body weight ratio was significantly larger than male splenic weight to body weight ratio. These trends noted for DES-induced splenic weight changes are similar to the DES effect on splenic lymphocyte cell count (**Figure 4.3**). The splenocyte count in adult males was not affected by the prenatal treatment of oil or DES. Females, on the other hand, had a significant decrease in splenocyte count when exposed prenatally to DES compared to their oil treated gender matched controls. We next investigated whether the decrease in splenocyte numbers in DES treated females was due to a decrease in T-cell populations.

Effects of prenatal exposure to DES on splenic T-cell populations

Splenocytes collected immediately after harvesting were stained for cytometric analysis of T-cell subsets. There was no significant change in the relative percentages or total number of T-cell subsets (CD4$^+$CD8$^-$, CD4$^-$CD8$^-$, CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$) in either oil or DES treated mice (**Table 4.2A and 4.2B**). Since there was a significant decrease in the total number of splenocytes in females given DES, we also notice an overall non-significant decreasing trend in the total number of T cells in the spleen of DES treated females. Splenic T-cells were also analyzed for apoptosis using 7AAD, a DNA binding dye. We found that there were no significant changes in the relative percentages of apoptotic T-cells

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in the spleen of oil or DES treated mice (data not shown). Since there were no changes in the T cell population count, it is likely that DES induced production of IFNγ in response to *in-vivo* and *in-vitro* stimulations is due to altered T cell functions.

Effects of prenatal exposure to DES on splenic T cell functionality

CD28 expression is an important co-stimulator for T-cell activation and production of the master Th-1 type cytokine, IFNγ. **Figure 4.4** shows that upon Con-A stimulation there is a significant increase in expression of CD28 on T cells in DES treated female mice. There were no significant differences in CD28 expression on T-cells from male mice given DES or oil treatments.

Effects of prenatal exposure to DES on the development of autoimmunity

Salivary glands, kidney and thyroid were examined for elevated lymphocyte infiltration, which is characteristic of autoimmune disease. Both prenatal treatments (oil and DES prenatally exposed males and females) had lymphocytic sialoadenitis (inflammation of salivary glands) and mild pyelitis (inflammation of renal pelvis). The thyroid was essentially normal in all mice.

Splenocytes were analyzed for $CD4^+CD25^+$ cells also known as regulatory cells. These cells consisted of only 1 – 2% of the total splenic cellularity. There was no significant difference in the relative percentage of regulatory cells in DES or oil treated mice (Female$_{oil}$ 1.5 ± 0.3, Female$_{DES}$ 1.8 ± 0.3, Male$_{oil}$ 1.2 ± 0.3, Male$_{DES}$ 1.4 ± 0.3). Splenocytes cultured in media or ConA for 24 hours were stained for

CD4$^+$CD25$^+$ cells. **Table 4.3** shows the relative percentages of CD4$^+$CD25$^+$

populations in oil and DES treated mice. Although there were no significant

changes in CD4$^+$CD25$^+$ cells between oil or DES treatments, splenocytes from

male mice produced more CD4$^+$CD25$^+$ cells in response to ConA stimulation

than females.

Autoreactive lymphocytes in the spleen were also examined with cytometric

analysis. Autoreactive T-cells (TCRVβ8+) consisted of 1 – 3% of the total splenic

cellularity. Prenatal exposure to DES did not affect the relative percentage of

autoreactive T-cells (Female$_{oil}$ 1.8 $\pm$ 0.5, Female$_{DES}$ 1.8 $\pm$ 0.5, Male$_{oil}$ 2.8 $\pm$ 0.4,

Male$_{DES}$ 1.8 $\pm$ 0.5). Autoreactive B-cells (CD19$^+$CD40 ligand$^+$) also made a small

percentage of total splenic lymphocytes (1.3 $\pm$ 0.2% in DES treated mice and 1.2

$\pm$ 0.2% in oil treated mice).

Discussion

Diethylstilbestrol was the first synthetic hormone administered for therapeutic

purposes. Endocrine therapy has since been expanding. Natural and synthetic

estrogenic hormones have played a wide role in the treatment of reproductive

disorders such as breast cancer and prostate cancer as well as contraceptives

(ethinyl estradiol), menopausal and osteopathic therapies (hormone replacement

therapy). Despite the known reproductive and immunological side effects of DES

administration, hormone therapy is still used in clinical practices. Currently, there

has been a resurrection of hormone therapies used for the stabilization of high-risk pregnancies. Administration of progesterone, an endogenous hormone secreted by the ovaries, during pregnancy was shown to reduce the risk of premature births. DES was primarily administered to pregnant women for the same reason. Unfortunately, DES caused more harm than good. The reproductive abnormalities from prenatal DES exposure are well characterized. However, immunological abnormalities are only beginning to be recognized. There are few studies that explore the immunological effects of prenatal exposure to DES. Furthermore, there are fewer studies that focus on the immunological effects in both genders, simultaneously. This study was designed to address these gaps in the literature.

We administered low doses of DES to pregnant mice in order to simulate the realistic levels of exposure to environmental estrogens. Although the low dose (0.25μg) was too small to induce any appreciable effects on the reproductive organs, 0.25μg of DES was enough to induce significant immunological abnormalities later in life. Splenic lymphocyte cellularity and functionality was affected by DES exposure in female mice and not in males.

DES treated females had a significant decrease in splenocyte cellularity. However, the relative percentages of T and B cells were not affected. This suggests that females given DES during fetal development had an overall low lymphocyte count in the spleen. Similar results were reported in our previous

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study (Karpuzoglu-Sahin et al., 2001). As expected the majority of T-cell subsets

(**Table 4.2**), which have migrated to the spleen, are mature CD4$^+$CD8$^-$ and CD4$^-$

CD8$^+$ cells. There are virtually no immature CD4$^+$CD8$^+$ T-cells. The relative

percentage of CD4$^-$CD8$^-$ double negative cells was high because these

percentages include the B-cells, macrophages, dendritic cells and all other non-

T-cell lymphocytes normally found in the spleen.

When splenocytes were cultured with ConA, females treated with DES became

more stimulated than their controls. Splenocytes from female treated mice

produced higher levels of IFNγ and expressed more CD28 than oil treated

controls. Similar findings were reported in our previous study where mice, which

were exposed to a similar DES dose regimen (prenatally exposed to DES

followed by a secondary DES exposure later in life), had significantly higher

levels of secreted IFNγ and increased number of IFNγ producing cells compared

to negative controls (Karpuzoglu-Sahin et al., 2001). Likewise, in this study,

female DES treated mice responded with significantly higher levels of IFNγ in the

serum after an antigenic challenge compared to their controls. Males had a non-

significant decrease in IFNγ serum levels. This would suggest that females

prenatally exposed to DES have a highly stimulated immune response to foreign

antigens. Other studies have shown that mice given hormone treatments such

as hexoestrol in gonadectomized mice and administrations of pharmacological

concentrations of DES increased susceptibility to *T. gondii* infection (Kittas and

Henry, 1980, Pung and Luster, 1986).   This is the first study that shows prenatal

exposure to DES can have long-lasting effects on the immune response to foreign antigens *in-vivo*. It is possible that the hypersensitive immune response at early onset of exposure (six hours after administration of *T. gondii* antigens) may prematurely deplete the immune system of vital cells/activity before it is able to clear the antigen/infection completely, thus resulting in increased susceptibility to infection as reported in the previous papers.

This type of DES induced hypersensitivity may potentially lead to the development of autoimmunity. We investigated whether these mice showed any physiological characteristics of autoimmunity such as lymphocyte infiltration of salivary glands, kidneys and thyroid. Since these mice were already aged over one year, it is not uncommon to see lymphocytic sialoadenitis and pyelitis. Prenatal DES treatment did not play a role in the inflammation of these organs. We also looked at CD4+CD25+ regulatory T cells which have been associated with autoimmunity. Again, since we did not observe any DES influences on the relative percentage of this lymphocyte population, seems unlikely that DES treatment plays any role in the development of autoimmunity in these mice.

Immunological changes induced by prenatal exposure to DES are gender-specific. In this study, we report that females exposed to DES during fetal development have hypersensitive immune responses to *in-vitro* and *in-vivo* stimulants, and altered splenic T cell functions. Males did not present such severe immunological changes due to prenatal exposure to DES, as did females.

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IFNg levels in serum six hours after *Toxoplasma gondii* antigen challenge
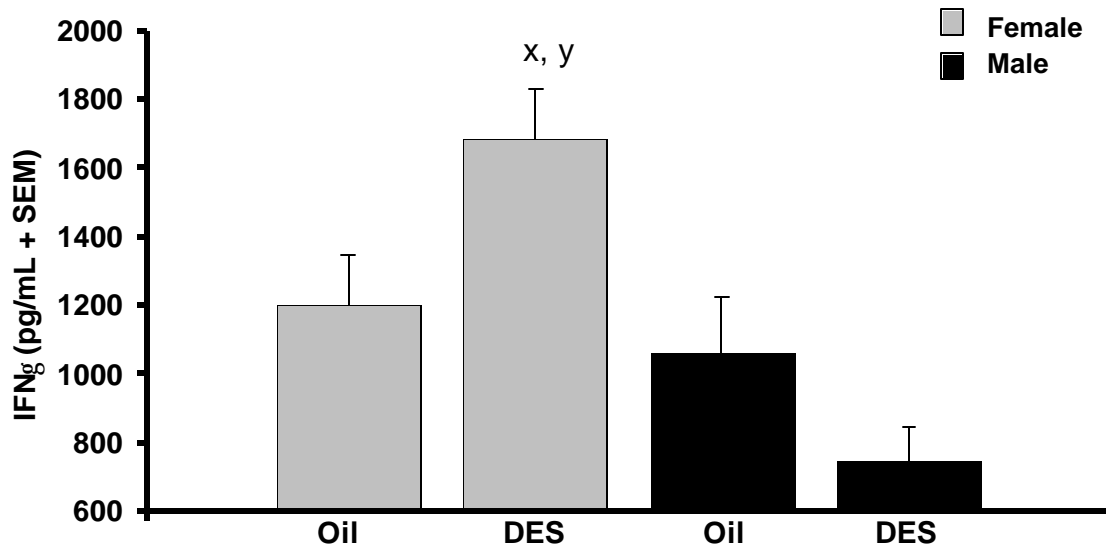
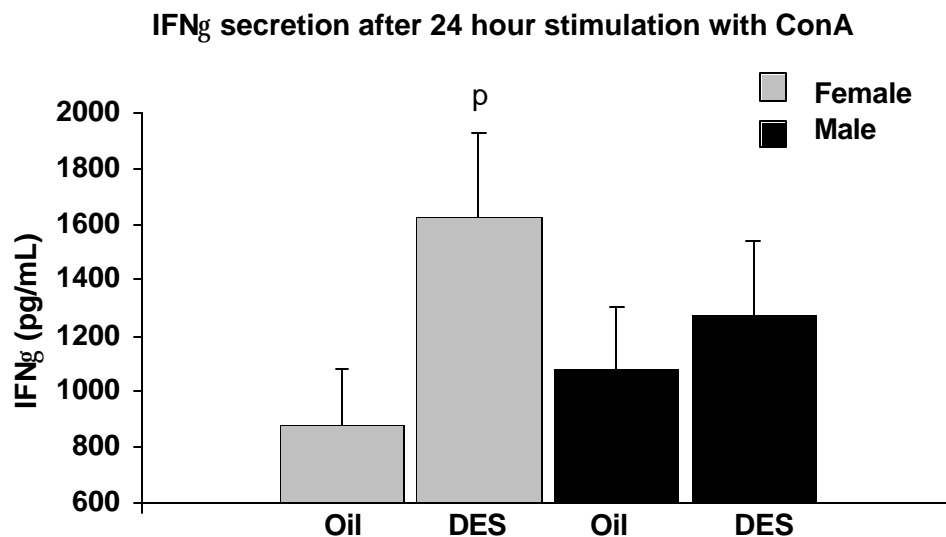
x denotes significant difference between female mice given DES and female mice given oil prenatal treatment ($P< 0.05$)
y denotes significant difference between female mice given DES and male mice given DES prenatal treatment ($P< 0.05$)

Figure 4.1 IFNg levels in serum six hours after *T. gondii* antigen challenge
Mice were prenatally exposed to either DES (0.25µg) or oil-only on the 14th day of gestation. One year later, mice were given one subcutaneous injection of (30µg/kg bw). Mice were then challenged with 20µg of the R.H. strain of *T. gondii* soluble antigen mixture. Serum samples were collected 6 hours after the *T. gondii* antigen challenge. Mean data are presented in pg/mL + standard error of the mean. Differences between genders and treatment groups were considered significant when $P<0.05$.

IFNg secretion after 24 hour stimulation with ConA

p denotes significant difference between females treated with DES prenatally and their oil treated controls

Figure 4.2 Secreted IFNg levels after 24-hour stimulation with ConA
C57BL/6 mice were exposed to either DES (0.25µg) (n_{female}= 7, n_{male}= 11) or oil (n_{female} = 10, n_{male}= 8) during prenatal development. At one year of age all mice were given 30µg/kgbw DES. Splenocytes were cultured with an optimal concentration of concanavalin-A (ConA; 10µg/mL). The supernatants were collected after 24 hours of culture and analyzed for IFNγ levels by ELISA. Mean data are represented in pg/mL + standard error of the mean. Differences between gender and prenatal treatments were considered significant when $P<0.05$.
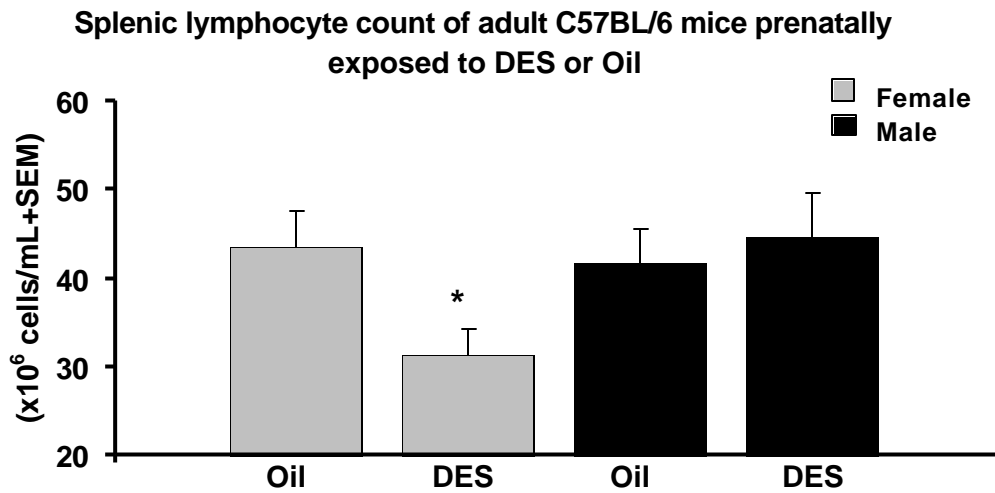
Splenic lymphocyte count of adult C57BL/6 mice prenatally exposed to DES or Oil

* denotes a significant difference between females given oil and females given DES

Figure 4.3 Splenic lymphocyte count of adult C57BL/6 mice prenatally exposed to DES or oil

C57BL/6 mice were exposed to either DES (0.25μg) (n_{female}= 7, n_{male}= 11) or oil (n_{female} = 10, n_{male}= 8) during prenatal development. At one year of age all mice were given 30μg/kgbw DES. Immediately after harvesting the spleen, splenocyte suspensions were counted on a Casy-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany). Data presented are mean + standard error of the mean. Differences between gender and prenatal treatments were considered significant when P<0.05.

Media   ConA

Oil

CD28+CD90+
45.7%

CD28+CD90+
20.4%

DES

CD28+CD90+
51.7%
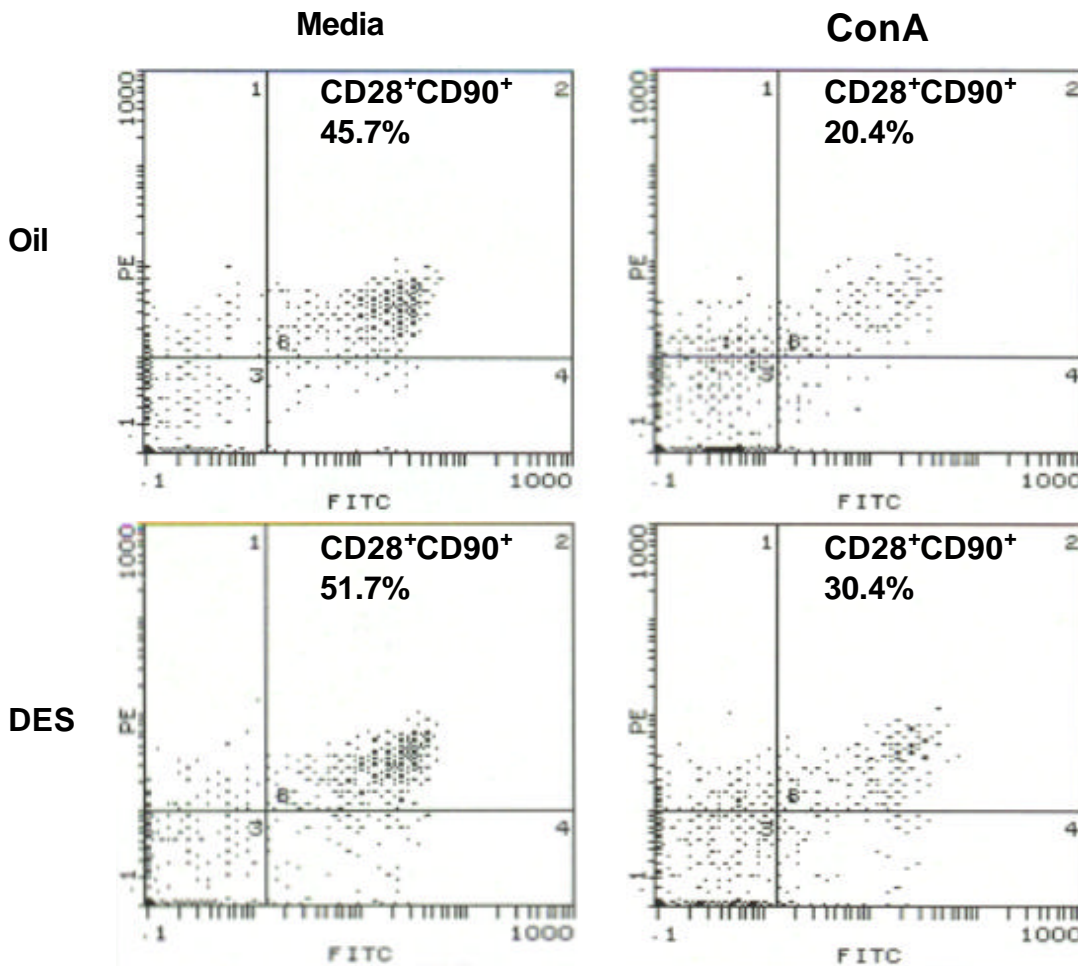
CD28+CD90+
30.4%

**CD28⁺CD90⁺ stimulated splenocytes from adult female
mice given prenatal oil or DES treatment**
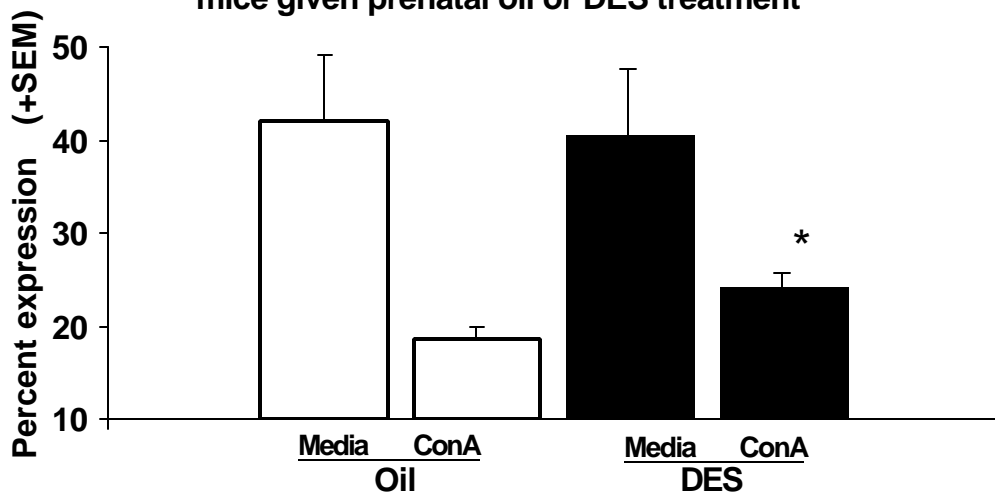
* denotes a significant difference between splenocytes from Oil and DES
prenatally treated female mice stimulated with ConA.

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Figure 4.4 CD28$^+$CD90$^+$ ConA stimulated splenocytes from mice given prenatal oil or DES treatment.

C57BL/6 mice were exposed to either DES (0.25?g) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development. At one year of age all mice were given 30?g/kgbw DES. Splenocytes were stained for CD28 co-stimulatory marker expressed on T cells (CD90). The data are presented as mean + standard error of the mean of CD28 expression on Tcells cultured in ConA for 24 hours. The relative percentages of CD28 expression on Tcells in media cultures included (Female$_{\text{oil}}$ =49.4 $\pm$ 7.7, Female$_{\text{DES}}$ =40.7 $\pm$ 7.1, Male$_{\text{oil}}$ =49.4 $\pm$ 7.0, Male$_{\text{DES}}$ =48.5 $\pm$ 7.3). Differences between genders and prenatal treatment were considered significant when $P<0.05$.

Table 4.1 Body weights and reproductive organ weights of adult C57BL6 female and male mice given either DES treatment or oil only control treatment during prenatal development

Organ	Gender	Oil	DES
Terminal Body Weight			
	Female	24.5 ± 1.1	25.3 ± 1.3
	Male	34.1 ± 1.3	34.5 ± 1.2
Reproductive Organs			
Uterus Weight (g ± SEM)	Female	0.10 ± 0.02	0.12 ± 0.02
Uterine Weight : Body Weight x 100 (± SEM)		0.41 ± 0.06	0.47 ± 0.07
Ovaries Weight (g ± SEM)	Female	0.01 ± 0.001	0.02 ± 0.001
Ovaries Weight : Body Weight x 100 (± SEM)		0.06 ± 0.004	0.06 ± 0.005
Seminal Vesicules Weight (g ± SEM)	Male	0.53 ± 0.07	0.60 ± 0.06
Seminal Vesicules : Body Weight x 100 (± SEM)		1.57 ± 0.22	1.74 ± 0.20
Testes Weight (g ± SEM)	Male	0.20 ± 0.006	0.19 ± 0.005
Testes Weight : Body Weight x 100 (± SEM)		0.56 ± 0.03	0.59 ± 0.02

C57BL/6 mice were exposed to either DES (0.25μg) (n_{female}= 7, n_{male}= 11) or oil (n_{female} = 10, n_{male}= 8) during prenatal development. At one year of age all mice were given 30μg/kgbw DES. Body weight and reproductive organ weight were recorded immediately after termination. The data are presented as mean ± standard error of the mean.

Table 4.2A anti-CD4-PE, anti-CD8-FITC stained splenocytes from DES or oil prenatally exposed mice immediately after harvest

	Female		Male	
Splenic T-cell Subsets	Oil	DES	Oil	DES
$CD4^+CD8^-$	21.5 ± 2.3	20.9 ± 2.0	18.5 ± 2.3	20.2 ± 2.0
$CD4^-CD8^+$	8.3 ± 2.0	8.6 ± 1.8	10.5 ± 2.0	9.8 ± 1.8
$CD4^+CD8^+$	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	1.0 ± 0.2
$CD4^-CD8^-$	69.1 ± 4.3	69.4 ± 3.7	69.9 ± 4.3	68.5 ± 3.7

Table 4.2B Splenic T cells (x10^6/spleen)

	Female		Male	
Splenic B-cell	Oil	DES	Oil	DES
$CD4^+CD8^-$	9.6 ± 2.0	7.2 ± 1.8	8.3 ± 1.8	8.8 ± 1.6
$CD4^-CD8^+$	3.8 ± 1.2	2.9 ± 1.0	4.7 ± 1.3	4.1 ± 1.2
$CD4^+CD8^+$	0.5 ± 0.04	0.4 ± 0.06	0.5 ± 0.07	0.04 ± 0.06
$CD4^-CD8^-$	29.7 ± 3.3	21.8 ± 3.4	29.2 ± 3.0	34.7 ± 3.5

C57BL/6 mice were exposed to either DES (0.25μg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development. At one year of age all mice were given 30μg/kgbw DES. Splenocytes were stained for T-cell subset markers CD4 and CD8 immediately after harvest. The data are presented as mean ± standard error of the mean.

Table 4.3 Relative percentages of CD4$^+$CD25$^+$ splenocytes after 24-hour incubation from female and male mice exposed to either oil or DES prenatally.

Splenocyte Subset	Female Media Oil	Female Media DES	Female ConA Oil	Female ConA DES	Male Media Oil	Male Media DES	Male ConA Oil	Male ConA DES
CD4$^+$CD25^{+e}	1.5 ± 0.3	1.8 ± 0.4	14.3 ± 2.5	12.2 ± 2.2	0.9 ± 0.2	0.7 ± 0.3	23.3 ± 2.1	22.3 ± 2.4

[e] denotes a significant difference between female splenocytes cultured with ConA and male splenocytes cultured with ConA

C57BL/6 mice were exposed to either DES (0.25µg) (n_{female}= 6, n_{male}= 6) or oil (n_{female} = 6, n_{male}= 6) during prenatal development. At one year of age all mice were given 30µg/kgbw DES. Splenocytes were stained for regulatory T cells CD4$^+$CD25$^+$ immediately after harvest. The data are presented as mean $\pm$ standard error of the mean. Differences between gender and prenatal treatments were considered significant when $P<0.05$.

Chapter 5

Conclusions

Overall, we have learned that the synthetic estrogen, diethylstilbestrol, is not only a potent estrogenic compound but it is also a highly potent immunomodulator. We have found that doses too low to induce reproductive effects are sufficient to induce severe immunological effects. In the studies described, micrograms of DES were administered to both males and females. Chapter 2 showed that only the highest dose administered had detectable mild reproductive effects (decreased seminal vesicle weight and increase uterine epithelial cell height) while the lowest dose administered showed altered splenic lymphocyte gender-specific responses to T cells stimulants. Likewise, in Chapters 3 and 4 pregnant mice were exposed to 0.25µg of DES during one day of their gestational period. Considering that a pregnant mouse (which received 0.25µg of DES) weighs approximately 0.04kg, a pregnant woman, who may weigh approximately 72kg, given an equivalent dose would have received only 0.5mg of DES. This dose would fall in the lowest half of the wide range of doses prescribed during the 1940-1970s, yet we are noticing significant DES induced alterations in the immune system of prenatally exposed mice. It is only assumed that with higher doses of DES administered there would be more severe immune and reproductive alterations.

Since it is well known that estrogenic compounds e.g. DES affect the reproductive system, it is not surprising to notice that DES exposed people have

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high risk of developing reproductive disorders. Furthermore, both males and

females are at equal risk to developing the many DES related reproductive

abnormalities mentioned earlier. Interestingly, we found that males and females

are not at equal risk to developing the DES induced immunological alterations.

In fact, we report that the female immune system is far more sensitive to DES

than the male immune system. For instance, both prenatal and adult DES

exposed females were noticed to have altered immune response in both the

thymus and spleen compared to their gender matched controls. In contrast,

males prenatally exposed to DES did not show any immunological effects in their

lymphocyte functionality from the thymus or spleen. In addition, males exposed

to DES during adult life had seemingly delayed proliferative response to T cell

stimulants compared to females exposed to DES.

We have also shown that DES induced immunological effects differ based on the

time of exposure. Prenatal DES exposed females had a hypersensitive immune

response compared to adult DES exposed females had a seemingly suppressed

immune response to T cell *in-vitro* stimulants. On the other hand, males

prenatally exposed to DES had close to comparable immune responses to their

negative controls, yet adult DES exposed males had altered T-cell functions and

an increase in apoptotic thymocytes.

Through these studies we have reported that DES can induce immunological

changes in mice. However, these changes are specific to the dose administered

as well as the gender and age of the subject who is exposed. We emphasize the importance of these three conditions to be incorporated into experimental designs of future studies that focus on immunological consequences to hormone exposure.

Overall, these are the first studies to report that fetal exposure to DES has long-term immunological consequences that are evident as late as one year of age. Further, these studies are also novel in showing that immunological responses of males and females to DES are different. These studies suggest that the DES effects on the immune system cannot be generalized between genders since DES exposure may have varied effects in men and women.

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Virginia Maryland Regional College of Veterinary Medicine (VMRCVM),
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Minor: Chemistry

RESEARCH INTERESTS

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RESEARCH EXPERIENCE

Laboratory Technician, TechLab, Inc.,
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Graduate Student and Teacher Assistant, VMRCVM,
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-   Studied in the department of Immunology in the College of Veterinary
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Student Research Trainee, Uniformed Services University of the Health Sciences,
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- Studied proinflammatory cytokine expression in various organs of hemorrhagic shock rats pretreated with anti-inflammatory drugs, curcumin and picroliv.

<u>Undergraduate Research</u>, Department of Plant Pathology, Virginia Tech, Blacksburg, VA, Jan. 1999-May 1999

- Studied the sensitivity of Rsv, soybean mosaic virus resistance genes, under heat controlled conditions by immunological detection of viral antigen, comparing soybean phenotypes, and DNA fragmentation electrophoresis indicative of cell apoptosis
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<u>Laboratory Assistant</u>, Department of Plant Pathology, Virginia Tech, Blacksburg, VA, Aug. 1998- Dec. 1998

- Assisted and learned laboratory techniques such as immunoblots, gel electrophoresis, double stranded RNA extraction, subculturing, mechanical virus inoculation

<u>Undergraduate Research</u>, Department of Microbiology, Virginia Tech, Blacksburg, VA, May 1998- Aug. 1998

- Tested for cadmium chloride resistance using disk sensitivity methods of several Gluconobacter species.
- Prepared a variety of media, grew up stock collections, and performed general microbiology laboratory procedures

<u>Research Assistant</u>, Biological Sciences and Biotechnology Cross Cutting Initiative, Virginia Tech, Blacksburg, VA, May 1998- July 1998

- Researched biological sciences related programs at various universities via web-sites and e-mail
- Collected information was compiled into a report and submitted to the President and Deans of Virginia Tech

Presentations

1. **Calemine, J**. and Tolin, S. SMV-Soybean and Heat. 1999 American Society for Microbiology, Blacksburg, VA, November 5-6, 1999 .
2. Gaddipati, J. P., **Calemine, J**., Seth, P., Sidhu, G. S., and Maheshwari, R. K. Curcumin regulates cytokine expression during hemorrhagic shock and resuscitation in rats.Seventh Annual Conference, International Cytokine Society, Hilton Head Island,SC, December 5-9, 1999 .
3. Gaddipati, J. P., **Calemine, J**, Seth, P.,Sidhu, G. S., and Maheshwari, R. K. Pretreatment with curcumin modifies cytokine expression during hemorrhagic

shock and resuscitation in rats.39th Annual Meeting of The American Society for Cell Biology, Washington, DC, December 11-15, 1999 .

4. Ahmed, S.A., Verthelyi, D., Karpuzoglu-Sahin, E., **Calemine, J**. "Estrogenic hormones alter apoptosis and induce autoimmunity in normal mice." 5th International Congress of the Immunology of Diabetes Society. Madras, India. February 14-16, 2001

5. Ahmed, A.S., Karpuzoglu-Sahin, E., Hissong, B., **Calemine, J**. and Donner, K. " Estrogenic hormones (17β-estradiol and diethylstilbestrol) up-regulate interferon-gamma and nitric oxide." Experimental Biology 2001. Orlando, FL. March31-April 4, 2001.

6. Ahmed, A.S., Verthelyi, D., Karpuzoglu-Sahin, E., **Calemine, J**. "Estrogenic Hormones Promote B Cell Function: Induction of autoantibodies in normal mice." Experimental Biology 2001. Orlando, FL. March 31- April 4, 2001

7. **Calemine, J**., Karpuzoglu-Sahin, E., Lengi, A., and Ahmed, A.S. "Estrogenic compounds induce nitric oxide production and release in murine splenocytes" Abstract submitted to 13th Annual Research Symposium, June 2001, Blacksburg, VA.

8. Karpuzoglu-Sahin, E., **Calemine, J**, Lengi A., and Ahmed, A.S. "Studies in IFN-γ and iNOS knockout mice suggest that IFN-γ and NO play a central role in preventing activation-induced apoptosis." Abstract submitted to 13th Annual Research Symposium, June 2001, Blacksburg, VA.

9. Ansar Ahmed, S., Karpuzoglu-Sahin, E., **Calemine J**., and Lengi, A. "Estrogen, Immunity and Autoimmunty. B cells and Autoimmunity: New concepts and therapeutic perspectives." Satellite Meeting to the11[th] International Congress of Immunology, July 2001. Bergen, Norway.

Publications

1. **Calemine, J**., Gogal R.M, Lengi, A., Sponenberg P., Ahmed, A.S. "Immunomodulation by diethylstilbestrol is dose and gender related: Effects on thymocyte apoptosis and mitogen-induced proliferation." *Toxicology*. 178 (2002). 101-118.

2. Gaddipati JP, Sundar SV, **Calemine J**, Seth P, Sidhu GS, Maheshwari RK. Differential regulation of cytokines and transcription factors in liver by curcumin following hemorrhage/resuscitation. *Shock* 2003 Feb;19(2):150-6.

LICENSE

- Certified to work with radioactive materials by the Department of Environmental Health and Occupational Safety of the Uniformed Services University of Health Sciences.

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