

**Effects of Short-Term Exposure to Octylphenol and  
Genistein on the Immune System of C57BL/6 and  
(NZBxNZW)<sub>F1</sub> Mice**

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## **(ABSTRACT)**

Octylphenol and genistein are two of the growing list of endocrine disrupting chemicals found in the environment that mimic estrogen in reproductive tissue both *in vitro* and *in vivo*. It is well established that endogenous estrogens modulate not only the reproductive system, but also the immune system. However, the effects of many endocrine disrupting chemicals, such as octylphenol and genistein, on the immune system have yet to be determined. Preliminary studies on short-term treatment with genistein (0.6 mg) and octylphenol (10 mg) showed that the thymus of orchietomized (NZBxNZW)<sub>F1</sub> males is sensitive to these agents. Further studies focused on the effects of short-term treatment of octylphenol on the morphology and function of the thymus in adult, reproductively intact non-autoimmune C57BL/6 and pre-autoimmune (NZBxNZW)<sub>F1</sub> males. Oral dosing of 0.1 mg, 1 mg, or 10 mg of octylphenol 3 times a week for 3 weeks did not affect the morphology or function of the thymus as assessed by its weight, thymocyte cellularity, proportion of immature and mature thymocytes, level of apoptosis, apoptotic rates of stimulated thymocytes, and proportion of mature T cells in the spleen. Furthermore, oral dosing of 0.1 mg, 1 mg, or 10 mg of octylphenol did not result in estrogenic changes in the reproductive tract in our model. Subcutaneous injection of 10 mg of octylphenol resulted in skin lesions that confounded the assessment of its effects on the thymus. Further studies are needed to definitively determine the effects of octylphenol on the immune system of both males and females of various ages and to determine the effect of long-term exposure.

## **Dedication**

I would like to dedicate my thesis to Zoey who inspired me to start my graduate studies, to Casy and Cole who kept me entertained along the way, to Colter who kept me sane at the very end, and to Shawn who took care of me and these mongrels all along the way.

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## Table of Contents

	Page #
<b>Chapter 1: Introduction, Rationale, and Literature Review</b>	<b>1</b>
a.) Introduction and Rationale	1
b.) Literature Review	2
Estrogen	2
Structure and Function	2
Estrogen Receptor	3
Endocrine Disrupting Chemicals	3
Studies in Wildlife	4
Studies in Laboratory Animals	5
Studies in Humans	5
Octylphenol	5
Genistein	7
Estrogen and Estrogenic Chemicals and the Immune System	9
Gender Differences	9
Estrogenic Treatment	9
Estrogen and Estrogenic Chemicals and the Thymus	12
Thymus	12
Estrogenic Effects	12
Apoptosis	13
Estrogen and Estrogenic Chemicals and Autoimmune Disease	13
Gender Differences	13
Influences of Estrogen	14
Animal Models	14
(NZBxNZW) <sub>F1</sub> Model for SLE, (B/W)	16
<b>Chapter 2: Preliminary studies to determine the effects of short-term oral dosing of octylphenol and genistein on the thymus, spleen, and lymph nodes of C57BL/6 or B/W mice</b>	<b>19</b>
a.) Introduction	19
b.) Specific Aim	20
c.) Materials and Methods	20

<b>Table of Contents cont.</b>	<b>Page #</b>
d.) Results	24
e.) Discussion	27
<b>Chapter 3: Dose range studies to determine the effects of short-term oral administration of octylphenol on the thymus of reproductively mature C57BL/6 and B/W males</b>	<b>52</b>
a.) Introduction	52
b.) Specific Aims	53
c.) Materials and Methods	54
d.) Results	57
e.) Discussion	60
<b>Chapter 4: Route of administration studies to compare the effects of oral and subcutaneous administration of octylphenol and 17<math>\beta</math>-estradiol in C57BL/6 and B/W Mice</b>	<b>82</b>
a.) Introduction	82
b.) Specific Aims	83
c.) Materials and Methods	84
d.) Results	87
e.) Discussion	92
<b>Chapter 5: General Discussion</b>	<b>122</b>
<b>Literature Cited</b>	<b>126</b>
<b>Appendix</b>	<b>148</b>
<b>Vita</b>	<b>152</b>

## **List of Figures and Tables**

Fig 1.1	Estrogen Production in the Ovaries	18
Fig 2.1	Effects of Octylphenol Treatment on the Cellularity and Weight of Lymphoid Organs in Orchiectomized B/W Mice	30
Fig 2.2	Effect of Octylphenol Treatment on Thymocyte Subsets of Orchiectomized B/W Mice	31
Fig 2.3	Mitogen-Induced Proliferation of Thymocytes from Octylphenol Treated Orchiectomized B/W Mice	32
Fig 2.4	Activation-Induced Apoptosis of Thymocytes from Octylphenol Treated Orchiectomized B/W Mice	33
Fig 2.5	Effects of Octylphenol Treatment on the T cell, B cell, and Macrophage Subsets in Secondary Lymphoid Organs of Orchiectomized B/W Mice	34
Fig 2.6	Effects of Octylphenol Treatment on the Secretion of IFN- $\gamma$ from Splenic Lymphocytes of Orchiectomized B/W Mice Stimulated with Con A and PMA+I	35
Fig 2.7	Effects of Octylphenol Treatment on the Secretion of IFN- $\gamma$ by Lymphocytes from the Lymph Nodes of Orchiectomized B/W Mice Stimulated with Con A and PMA+I	36
Fig 2.8	Secretion of IL-2 by Stimulated Lymphocytes from the Spleen and Lymph Nodes of Octylphenol Treated Orchiectomized B/W Mice	37
Fig 2.9	Effect of Octylphenol Treatment on the Expression of Very Early Activation Antigen, CD69, on Stimulated Cells from Orchiectomized B/W Mice	38
Fig 2.10	Mitogen-Induced Proliferation of Splenic and Lymph Node Leukocytes from Octylphenol Treated Orchiectomized B/W Mice	39

## **List of Figures and Tables cont.**

Fig 2.11	Cell Cycle Analysis of Splenic Leukocytes from Octylphenol Treated Orchiectomized B/W Mice at 24 and 48 Hours of Culture	40
Fig 2.12	Cell Cycle Analysis of Leukocytes from the Lymph Nodes of Octylphenol Treated Orchiectomized B/W Mice at 24 and 48 Hours of Culture	41
Fig 2.13	Effect of Genistein Treatment on the Weight and Cellularity of Lymphoid Organs in C57BL/6 Mice	42
Fig 2.14	Effect of Genistein Treatment on the Thymocyte Subsets of C57BL/6 Mice	43
Fig 2.15	Mitogen-Induced Proliferation of Thymocytes from Genistein Treated C57BL/6 Mice	44
Fig 2.16	Activation-Induced Apoptosis of Thymocytes from Genistein Treated C57BL/6 Mice	45
Fig 2.17	Effects of Genistein Treatment on Leukocyte Subsets of the Spleen and Lymph Nodes of C57BL/6 Mice	46
Fig 2.18	Effects of Genistein Treatment on the Expression of Very Early Activation Antigen, CD69, in C57BL/6 Mice	47
Fig 2.19	Proliferation of Mitogen-Stimulated Leukocytes from the Spleen and Lymph Nodes of Genistein Treated C57BL/6 Mice	48
Fig 2.20	Cell Cycle Analysis of Splenic Leukocytes from Genistein Treated C57BL/6 Mice at 24 and 48 Hours of Culture	49
Fig 2.21	Cell Cycle Analysis of Leukocytes from the Lymph Nodes at 48 hours of Culture	50
Fig 2.22	Effect of Genistein Treatment on the Secretion of IL-2 and IFN- $\gamma$ from Lymphocytes of the Spleen and Lymph Nodes of C57BL/6 Mice	51



## List of Figures and Tables cont.

Fig 3.1	Effect of Octylphenol Treatment on the Percent Body Weight of the Thymus in B/W and C57BL/6 Mice	63
Fig 3.2	Effect of Octylphenol Treatment on Thymocyte Cellularity in B/W and C57BL/6 Mice	64
Fig 3.3	Effects of Octylphenol Treatment on the Proportion of Mature and Immature Thymocytes in B/W and C57BL/6 Mice	65
Fig 3.4	Effect of Octylphenol Treatment on Fresh Apoptosis of Thymocytes in B/W and C57BL/6 Mice	66
Fig 3.5	Effect of Octylphenol Treatment in B/W Mice on the Proportion of Live (7-AAD <sup>dull</sup> ) Thymocytes upon Anti-CD3 Stimulation	67
Fig 3.6	Effect of Octylphenol Treatment in B/W Mice on the Apoptosis of Thymocytes upon Anti-CD3 Stimulation	68
Fig 3.7	Effect of Octylphenol Treatment in C57BL/6 Mice on the Proportion of Live (7-AAD <sup>dull</sup> ) Thymocytes upon Anti-CD3 Stimulation	69
Fig 3.8	Effect of Octylphenol Treatment in C57BL/6 Mice on the Apoptosis of Thymocytes upon Anti-CD3 Stimulation	70
Fig 3.9	Effect of Octylphenol Treatment in B/W Mice on the Proportion of Live (7-AAD <sup>dull</sup> ) Thymocytes upon PMA+I Stimulation	71
Fig 3.10	Effect of Octylphenol Treatment in B/W Mice on the Apoptosis of Thymocytes upon PMA+I Stimulation	72
Fig 3.11	Effect of Octylphenol Treatment in C57BL/6 Mice on the Proportion of Live (7-AAD <sup>dull</sup> ) Thymocytes upon PMA+I Stimulation	73

### **List of Figures and Tables cont.**

Fig 3.12	Effect of Octylphenol Treatment in C57BL/6 Mice on the Apoptosis of Thymocytes upon PMA+I Stimulation	74
Fig 3.13	Effect of Octylphenol Treatment on the Cellularity of the Bone Marrow of B/W and C57BL/6 Mice	75
Fig 3.14	Effect of Octylphenol Treatment on the Level of Anti-dsDNA Autoantibodies in the Serum of B/W and C57BL/6 Mice	76
Fig 3.15	Effect of Octylphenol Treatment on the Percent Body Weight of the Spleen in B/W and C57BL/6 Mice	77
Fig 3.16	Effect of Octylphenol Treatment on the Leukocyte Cellularity of the Spleen in B/W and C57BL/6 Mice	78
Fig 3.17	Effect of Octylphenol Treatment on B and T cell Markers of Splenic Leukocytes in B/W and C57BL/6 Mice	79
Fig 3.18	Effects of Octylphenol Treatment on the Percent Body Weight of the Testis in B/W and C57BL/6 Mice	80
Fig 3.19	Effect of Octylphenol Treatment on the Percent Body Weight of the Seminal Vesicles in B/W and C57BL/6 Mice	81
Fig 4.1	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Cellularity of the Thymus in B/W and C57BL/6 Mice	97
Fig 4.2	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Thymus in B/W and C57BL/6 Mice	98
Fig 4.3	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Proportion of Mature and Immature Thymocytes in B/W and C57BL/6 Mice	99
Fig 4.4	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on Apoptosis of Freshly isolated Thymocytes in B/W and C57BL/6 Mice	100

## List of Figures and Tables cont.

Fig 4.5	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in B/W Mice on the Proportion of Live, (7-AAD <sup>dull</sup> ) Thymocytes upon Anti-CD3 Stimulation	101
Fig 4.6	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in B/W Mice on the Apoptosis of Thymocytes upon Anti-CD3 Stimulation	102
Fig 4.7	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in C57BL/6 Mice on the Proportion of Live, (7-AAD <sup>dull</sup> ) Thymocytes upon Anti-CD3 Stimulation	103
Fig 4.8	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in C57BL/6 Mice on the Apoptosis of Thymocytes upon Anti-CD3 Stimulation	104
Fig 4.9	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in B/W Mice on the Proportion of Live, (7-AAD <sup>dull</sup> ) Thymocytes upon PMA+I Stimulation	105
Fig 4.10	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in B/W Mice on the Apoptosis of Thymocytes upon PMA+I Stimulation	106
Fig 4.11	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in C57BL/6 Mice on the Proportion of Live, (7-AAD <sup>dull</sup> ) Thymocytes upon PMA+I Stimulation	107
Fig 4.12	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in C57BL/6 Mice on the Apoptosis of Thymocytes upon PMA+I Stimulation	108
Fig 4.13	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Cellularity of the Bone Marrow in B/W and C57BL/6 Mice	109
Fig 4.14	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Proportion of Developing Granulocytes, Macrophages, Monocytes in the Bone Marrow of B/W and C57BL/6 Mice	110

### **List of Figures and Tables cont.**

Fig 4.15	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Level of Anti-dsDNA Autoantibodies in the Serum of B/W and C57BL/6 Mice	111
Fig 4.16	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Spleen in B/W and C57BL/6 Mice	113
Fig 4.17	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Leukocyte Cellularity of the Spleen in B/W and C57BL/6 Mice	114
Fig 4.18	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the T and B Cell Markers of Splenic Leukocytes in B/W and C57BL/6 Mice	115
Fig 4.19	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Expression of CD71 on T and B cells from the Spleen of B/W and C57BL/6 Mice	116
Fig 4.20	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Proportion of GR1 <sup>+</sup> F480/CD11b <sup>+</sup> Developing Granulocytes, Macrophages, Monocytes, in the Spleen of B/W and C57BL/6 Mice	117
Fig 4.21	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in the Expression of Very Early Activation Antigen, CD69, on Splenic Leukocytes from B/W and C57BL/6 Mice	118
Fig 4.22	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Expression of CD69 and GR1 on Splenic Leukocytes from B/W and C57BL/6 Mice	119
Fig 4.23	Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Testis in B/W and C57BL/6 Mice	120

**List of Figures and Tables cont.**

Fig 4.24	Effect of Octylphenol and $17\beta$ -estradiol Treatment on the Percent Body Weight of the Seminal Vesicles in B/W and C57BL/6 Mice	121
Table 4.1	Subcutaneous Injection of 10 mg of Octylphenol Results in Hepatic Hematopoiesis in B/W and C57BL/6 Mice	112

## List of Abbreviations

7-AAD	7 aminoactinomycin D
ANOVA	analysis of variance
APEO	alkylphenol ethoxylates
BSA	bovine serum albumin
B/W	(NZBxNZW) <sub>F1</sub>
Con A	concanavalin A
DES	diethylstilbesterol
DDT	dichlorodophenyl-trichlorethane
DMSO	dimethylsulfoxide
E1	estrone
E2	estradiol
E3	estriol
EDC	endocrine disrupting chemical
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ER $\alpha$	estrogen receptor alpha
ER $\beta$	estrogen receptor beta
FITC	fluorescein isothiocyanate
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MBSA	methylated bovine serum albumin
MHC	major histocompatibility complex
NK	natural killer cells
NOD	non-obese diabetic mice
NZB	New Zealand black mice
NZW	New Zealand white mice
PCD	programmed cell death
PE	phycoerythrin
PHA	phytohaemagglutinin
PMA+I	phorbol myristate acetate + ionomycin
RBC	red blood cell
SNF <sub>1</sub>	(NZBxSWR) <sub>F1</sub>
SLE	systemic lupus erythematosus
ssDNA	single stranded deoxyribonucleic acid
TCR	T cell receptor
TNF	tumor necrosis factor

# Chapter 1

## Introduction, Rationale, and Literature Review

### a.) Introduction and Rationale

Estrogen affects not only the reproductive system, but also the immune, cardiovascular, and central nervous systems (Grossman, 1984; Ansar Ahmed et al., 1985a; Olsen and Kovacs, 1996; Beyer, 1999; Mendelsohn and Karas, 1999). Estrogen has been shown to induce thymic atrophy, alter B cell and T cell mediated immunity, and accelerate the onset of autoimmune disease (Grossman, 1984; Ansar Ahmed et al., 1985a; Olsen and Kovacs, 1996). Exposure to estrogens can occur through endogenous estrogen, such as 17 $\beta$ -estradiol; through synthetic compounds, such as diethylstilbesterol, oral contraceptives, or estrogen replacement therapy; or through environmental estrogenic compounds, such as pesticides, surfactants, phytoestrogens, and industrial chemicals. These environmental chemicals that have been shown to mimic estrogen in the body are called environmental estrogens. Environmental estrogens are part of a broader category of chemicals known as endocrine disrupting chemicals (EDC), which are able to disrupt the endocrine system by mimicking, antagonizing, or altering the signaling of any hormone. Octylphenol, a surfactant, and genistein, a phytoestrogen, are two EDCs that have been shown to mimic estrogen in reproductive tissue both *in vitro* and *in vivo* tissue (Mueller and Kim, 1978; Arts and Van Den Berg, 1989; Whitten et al., 1993; White et al., 1994; Levy et al., 1995; Blake and Boockfor, 1997; Blake and Boockfor, 1997; Coldham et al., 1997). Both octylphenol and genistein have also been shown to alter cellular responses in ways estrogen does not. For example, octylphenol was toxic to splenocytes *in vitro*, while estrogen was not, and genistein inhibits tyrosine kinase activity in an estrogen receptor independent manner (Piontek, et al., 1993; Boutin, 1994; Nair-Menon et al., 1996). Therefore, both of these compounds have the potential to effect the immune system through their estrogenic and non-estrogenic activity. Thus far, there are no reported studies on the short-term effects of octylphenol or genistein on the immune system. These initial studies attempt to bridge this gap in the literature. The aim of these studies is to determine the immunomodulatory effects of short-term exposure to octylphenol and genistein on adult male mice, focusing especially on the effects of octylphenol treatment on the thymus of normal (C57BL/6) and pre-autoimmune (NZBxNZW)<sub>F1</sub> reproductively mature male mice.

## **b) Literature Review**

### **Estrogen**

**Structure and Function:** Estrogen, like all steroid hormones is derived from the parent compound cholesterol. All steroid hormones are derived from a cyclopentanoperhydrophenanthrene nucleus. Estrogens, 18 carbon compounds (C18), are derived from the 19 carbon (C19) androgen compounds by the removal of the methyl group at carbon 10 leading to the characteristic aromatic ring A (Gower, 1979).

The ovaries are the main source of estrogen in pre-menopausal women, while the adrenals produce small amounts of estrone. In men the testis are a main source of estrogen. Furthermore, peripheral tissues are able to utilize plasma 4-androstenedione and testosterone to produce estrogen in men and women, and peripheral tissue is the main source of estrogen in post-menopausal women (Gower, 1979).

In the ovaries, the thecal and granulosa cells are the principle cells in which estrogen is synthesized from cholesterol(Fig. 1.1). Cholesterol is synthesized into the androgens: androstenedione and testosterone. The testosterone and androstenedione are then converted into estradiol (E2) and estrone (E1), respectively, by an aromatase. The cholesterol to androgen pathway occurs in the thecal cells. The androgens can then be transferred to the granulosa cells, transformed into estrogen, and the estrogen utilized locally. Alternatively, the androgens can be converted to estrogen in the thecal cells and released into circulation (Kettyl and Arky, 1998). The estrogens, estradiol and estrone, can be interconverted by 17 $\beta$ -hydroxysteroid oxidoreductase (Kettyl and Arky, 1989). Furthermore, estrone can then be hydrated to form estriol (E3) (Gower, 1979; Kettyl and Arky, 1989).

Estrogen is classified as a female sex hormone because it is required for the maturation of the female reproductive tract. It is necessary for the development of the vagina, uterus, and fallopian tubes and it stimulates the growth of the endometrial lining of the uterus, vaginal secretions, and cervical mucus. However, it also has many other functions in the body. It is involved in lipid metabolism, calcium metabolism, and blood coagulation (Kettyl and Arky, 1998). Furthermore, it modulates the central nervous system, cardiovascular system, and the immune system, and has been shown to be necessary for proper function of the male reproductive system in estrogen receptor knock-out mice (Hess et al., 1997; Couse and Korach, 1998).



**Estrogen Receptor:** Estrogen receptors are thought by some to be localized in the cytosol. Estrogen crosses the plasma membrane and binds to the cytoplasmic receptor in two steps. First a hydrogen bond is formed between the C3 hydroxyl group on estrogen and a side chain in the receptor-binding site. Then, estrogen is stabilized by a hydrogen bond between the D ring and the receptor (Raynaud et al., 1985; Anstead and Kym, 1995). The receptor-estrogen complex is then transported into the nucleus. In the nucleus, the receptor-estrogen complex is able to act as a nuclear transcription factor by binding to estrogen-response elements of the DNA. This binding can facilitate the assembly of transcription complexes, thus influencing the transcription of DNA. Two separate estrogen receptors have been identified and characterized, estrogen receptor-alpha ( $ER\alpha$ ) and estrogen receptor-beta ( $ER\beta$ ). In humans the DNA-binding domain and the ligand binding domains are highly conserved between  $ER\alpha$  and  $ER\beta$ . However, the hinge region, F domain, and A/B domains of the receptors are not conserved (Mosselman et al., 1996).

Estrogen receptors are not only localized to reproductive tissue, but are also found in immune cells and other tissue.  $ER\beta$  has been found in the ovaries, testis, spleen and thymus of adult humans (Mosselman et al., 1996) and ovaries, testis, adrenals, spleen, thymus, pituitary gland, skin, lung, kidney, and brain cortex in the human fetus (Brandenberger et al., 1997).  $ER\alpha$  has also been found not only in reproductive tissues such as the ovaries, uterus, and testis, but also in the spleen (Brandenburger et al., 1997). In the thymus, the ER (presumably  $ER\alpha$ ) has been localized to epithelial cells (Grossman et al., 1979; Nilsson et al., 1986; Kawashima et al., 1991), lymphocytes (Kawashima et al., 1992), and lymphoblastoid cells (Gulino et al., 1985). Some studies have linked thymulin production and estrogen receptors to the same epithelial cells and suggest that thymulin production, which stimulates thymocyte differentiation and maturation, is a mechanism for sex hormone regulation of the immune system (Sakabe et al., 1990; Kawashima et al., 1991). In the spleen, the ER (presumably  $ER\alpha$ ) has been localized the CD8+ T lymphocytes (suppressor/cytotoxic T cells) subpopulation, but not to CD4+ T lymphocytes (helper T cells) subpopulation (Cohen et al., 1983; Ansar Ahmed, 1985; Stimson, 1988). Furthermore, the estrogen receptor has been found in blood mononuclear cells (Weusten et al., 1986).

### **Endocrine Disrupting Chemicals**

Estrogens exist not only as natural compounds in our body or synthetic compounds for therapeutics use, but also as endocrine disrupting chemicals in the environment. Endocrine disruptors are abundant in the environment and

many compounds are continually being added to this category. They can be found in detergents and surfactants (Octylphenol, Nonylphenol), plants as phytoestrogens (Genistein, Coumesterol), pesticides (Dichlorodophenyl-trichloroethane (DDT), Methoxychlor, Dieldrin, Toxaphene), industrial chemicals (polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)), plastics (Bisphenol A, Octylphenol), and in fungal metabolites (Zearalenone).

A subset of EDCs act through the estrogen receptor and mimic estrogen. They are classified by some as environmental estrogens. Many of these EDCs are not structurally related to 17- $\beta$ -estradiol, an endogenous estrogen. Nevertheless, they exhibit estrogenic effects both *in vitro* and *in vivo*. They are able to bind to the estrogen receptor, translocate the receptor from the cytoplasm to the nucleus, bind to estrogen response elements, and induce the synthesis of estrogen receptor-regulated proteins (Danzo, 1998; Gray, 1998). Many of the environmental estrogens, such as the alkylphenols, methoxychlor, DDT, bisphenol A, and chlordecone, compete with 17 $\beta$ -estradiol for the binding of both ER $\alpha$  and ER $\beta$  with a similar preference and degree (Kuiper et al, 1998). In contrast, some of the phytoestrogens, such as genistein, coumesterol, apigenin, naringenin, and kaempferol, compete stronger with 17 $\beta$ -estradiol for the binding of ER $\beta$  than ER $\alpha$  (Kuiper et al, 1998). Environmental estrogens are also able to bind to hormone binding proteins, generally with lower binding affinities than estrogen (Nagel et al, 1998, 1999). However, because the environmental estrogens have lower binding affinities for the hormone binding proteins, they have enhanced access to estrogen receptor in serum (Nagel et al, 1998, 1999). *In vivo*, they increase uterine weights, reduce pregnancy rates and litter sizes, and alter the development of the reproductive tract and sexual behavior (Cummings, 1997). The EDCs are generally less potent than 17- $\beta$ -estradiol; however, they are generally more stable in the environment and have been shown to accumulate in body fat (Zhu and Norstrom, 1993). Furthermore, progeny may be exposed during pregnancy or through colostrum/milk intake. The global distribution of EDC exposure is evident from the observation that polar bears, seals, and humans in the arctic geographic region have elevated levels of EDCs in their body fat, even though no direct exposure has been noted (Zhu and Norstrom, 1993).

**Studies in Wildlife:** Studies on wildlife populations have shown reproductive changes associated with exposure to endocrine disrupting chemicals. In the Great Lakes, which is polluted with endocrine disrupting chemicals, they have noted feminization, decreased fertility, diminished hatching rates, and aberrant sexual behavior in the fish and birds (Fry and Toone, 1981;

Morrison et al., 1985). Furthermore, reproductive tract abnormalities have led to a decrease in progeny of alligators in Lake Apopka, Florida, which is also contaminated with endocrine disrupting chemicals (Guillette et al., 1994).

**Studies in Laboratory Animals:** Extensive studies in laboratory animals have clearly shown the effects of EDCs on the reproductive system and other tissues. Administration of methoxychlor, an estrogenic compound in pesticides, causes increased reproductive tract weight, hypertrophy of epithelial cells in the vagina and the uterus, and an increase in gestation period in rodents (Swartz and Corkern, 1992; Walters et al., 1993; Swartz et al., 1994). Premature aging of the mammary gland was noted in rats exposed to nonylphenol, bisphenol A, estrone, or diethylstilbesterol (DES) (Holland and Roy, 1995, 1996; Colerangle and Roy, 1995a, 1995b, 1997). Also, it has been shown that p,p'-DDE, an estrogenic metabolite of DDT, inhibits the effects of androgens in adult, prepubertal, and developing rats by blocking the binding of androgen to the androgen receptor and thus the transcriptional activity induced by androgens (Kelce et al., 1995). In other tissues, it has been shown that bisphenol A results in a higher incidence of multinucleated hepatocellular giant cells in male mice (Nat Tox Program, 1982).

**Studies in Humans:** Due to EDC's adverse effects on laboratory animals and their association with reproductive changes in wildlife, a linkage between EDC and adverse health effects in humans is being investigated. These estrogenic chemicals are suspected in the increase in the incidence of testicular cancer, hypospadias, and cryptorchidism (Toppari et al., 1996); decreased quality of sperm; decline in sperm counts (Carlsen et al., 1992); and increased incidence of breast cancer in women (Bradlow et al, 1996). *In vitro*, DES, nonylphenol, and bisphenol A result in telomeric associations and chromatid breaks in the human breast cancer cell line, MCF-7 (Banerjee and Roy, 1996). Furthermore, *in vivo*, prenatal exposure to DES is associated with increased incidence of vaginal adenocarcinomas (Herbst et al., 1971). The duration of lactation in women has been shown to decrease as the concentration of DDE in the milk increases (Rogan et al., 1987; Gladen and Rogan, 1995), and neurotoxicologic effects were reported in children exposed to PCBs (OTA, 1990).

**Octylphenol:** Alkylphenol ethoxylates (APEO) are precursors to alkylphenols, such as octylphenol and nonylphenol, which are classified as endocrine disrupting chemicals. APEOS are nonionic surfactants that comprise 25% of the total nonionic surfactant production in the United States (Nimrod and Benson, 1996). It was estimated that 300,00-600,00 tons/year of APEOs are produced worldwide (Chemical Manufacturers Assoc., 1994). They are found in industrial and domestic detergents, paints, pesticides, herbicides, laboratory

reagents, and shampoos (Giger et al., 1984; White et al., 1994; Cserhati, 1995). Alkylphenol ethoxylates are broken down slowly by progressive shortening of the ethoxylate chain to form alkylphenoxyacetic acids, alkylphenol monoethoxylates, and finally alkylphenols, such as octylphenol and nonylphenol (Giger et al., 1984). Alkylphenols have been found as breakdown products of APEOs in effluent from sewage treatment plants and in the aquatic environment such as river sediment. Furthermore, alkylphenols were detected in rats exposed to APEOs, suggesting the breakdown of APEOs can occur in the body (Knaak et al., 1966; Giger et al., 1984). Not only are alkylphenols breakdown products of APEOs, but they are also mass produced to be used in the production of APEOs and as antioxidants in clear plastics. Alkylphenols are degraded slowly and are able to bioaccumulate because of their lipid solubility (Nimrod and Benson, 1996).

Since the 1930s, it has been known that alkylphenols are estrogenic, and recent *in vitro* studies have found 4-*tert*-octylphenol (octylphenol) to be the most potent estrogenic alkylphenol (White et al., 1994). *In vitro*, octylphenol stimulates proliferation of human breast cancer cells and chicken embryonic fibroblasts transfected with the murine estrogen receptor, stimulates production of estrogen-responsive vitellogenin in male trout hepatocytes, and increases the expression of the estrogen-responsive prolactin gene in rat anterior pituitary cells (Mueller and Kim 1978; Soto et al., 1992; Jobling and Sumpter, 1993; White et al., 1994; Abraham and Frawley, 1997; Nagel et al., 1997). Octylphenol had been shown to mediate its estrogenic effects through the estrogen receptor binding to a similar region as 17- $\beta$ -estradiol (White et al., 1994). Octylphenol also has effects on the cardiovascular system, similar to endogenous estrogen. It has been demonstrated that *in vitro* octylphenol is able to inhibit the L-type  $\text{Ca}^{2+}$  channels in vascular smooth muscle cells and evoke rapid, endothelium-independent relaxation of coronary vasculature similar to estrogen (Ruehlmann et al., 1998). Octylphenol stimulates estrogen-dependent uterine growth in prepubertal rats and stimulates vitellogenin synthesis in male trout with concomitant inhibition of testicular growth (Bicknell et al., 1995; Jobling et al., 1995). Adult male rats exposed to octylphenol had decreased hematocrit, food consumption, and body weight gain and increased anterior pituitary/body weight ratios and prolactin levels (Blake and Boockfor, 1997). An extension of these studies showed octylphenol has adverse effects on the male reproductive system. This includes a decrease in sperm counts with an increase in tail and head abnormalities and an adverse effect on the size, weight, and histological structure of the testis, epididymides, ventral prostate gland, seminal vesicles, and coagulating gland (Boockfor and Blake, 1997). Furthermore, prenatal exposure to octylphenol has resulted in a decrease in testicular size and daily sperm production and a

decrease in the mRNA and protein levels of p450c17 in fetal leydig cells which is necessary for testosterone production (Sharpe et al., 1995, Majdic et al., 1996).

**Genistein:** Genistein is a phytoestrogen that is found in soy, alfalfa, clover, and other plants. By definition, phytoestrogens are compounds that are structurally or functionally similar to estrogen or are able to produce estrogenic effects. They are, therefore, classified as endocrine disrupting chemicals. Phytoestrogens are categorized as either lignans or isoflavonoids. The isoflavonoids are then divided into three sub-categories: isoflavones, isoflavans, or coumestans. Genistein is an isoflavone and comes from the plant precursor formononetin. Soybeans contain ~0.2-1.6 mg/g dry of isoflavones and clover can contain up to 5% dry weight of isoflavones (Adams, 1995; Kurzer and Xia, 1997). Also, alfalfa contains a small amount of isoflavones, and whole grain, potatoes, fruits, and vegetables all contain detectable levels of isoflavonoids, with isoflavones as the most prevalent group (Adams, 1995; Adlecreutz and Mazur, 1997).

In 1946, Bennetts et. al. first described the clinical manifestation of overexposure to isoflavones, specifically clover. Ewes that grazed in clover-rich fields exhibited prolapsed uteri, low lambing rates, enlargement of the bulbo-urethral glands, sterility, and death (Bennetts et al., 1946). Since then, many other cases have been reported. Heifers grazing in red clover rich fields developed 35% longer teats and exhibited an accumulation of fluid in the vagina and uterus (Nwannenna et al., 1994). Furthermore, ovarian cysts, irregular estrus, and anestrus have been noted in cattle exposed to phytoestrogens (Adams, 1995). The level of phytoestrogens in the forbs, a plant found in the breeding grounds of California quail, was shown to effect the breeding of the quail (Leopold et al., 1976). In dry years, the forbs contained significantly higher levels of genistein and formononetin than in the wet years, and higher levels of genistein and formononetin led to decreased breeding (Leopold et al., 1976). High levels of genistein and daidzein, another isoflavone, were also linked to high mortality due to liver problems and low fertility in North American captive cheetahs (Setchell et al., 1987). The commercial diet fed to the cheetahs contained 5-13% soy protein, which led to ~1 mg/kg/day of phytoestrogens. When the diet was changed to a chicken based diet, the liver conditions improved, thus mortality decreased, and females became pregnant (Setchell et al., 1987). Wildlife and livestock are not the only animals exposed to such high levels of phytoestrogens. Studies have shown that infants fed only soy formula may consume 6-9 mg/kg/day and adult vegetarians and Asians consume 0.3-1.2 mg/kg/day on average of phytoestrogens (Barnes, 1995; Setchell et al., 1997).

Both *in vitro* and *in vivo* studies have demonstrated the estrogenicity of genistein. Based on *in vitro* binding affinity assays with ER $\alpha$ , genistein is the second most estrogenic phytoestrogen, the most estrogenic being coumesterol (Arts and Van Den Burg, 1989; Coldham et al., 1997). However, binding affinity assays utilizing ER $\beta$  have found that coumesterol and genistein compete equally with 17 $\beta$ -estradiol for ER $\beta$  binding (Kuiper et al, 1998). Genistein has also been shown to be estrogenic *in vivo* in laboratory animals. In rodents genistein caused cornification, metaplasia, and delayed opening of the vagina (Whitten et al., 1993; Levy et al., 1995) and increased duct development in mammary tissue (Lamartiniere et al., 1995). Furthermore, genistein decreased birth weights, increased the ano-genital distance in males (Levy et al., 1995), altered lutenizing hormone levels (Faber and Hughes, 1991), and enlarged the sexually dimorphic nucleus in the preoptic area of the hypothalamus (Faber and Hughes, 1991).

Genistein is also considered a chemopreventative agent for breast cancer. Genistein is able to prevent the development of chemically or irradiation-induced tumors in mammary of mice (Messina et al, 1994). This may seem contradictory to the estrogenic effects of genistein; however, in breast cancer cell lines, genistein has been shown to have biphasic effects. Above 10  $\mu$ M genistein has an antiproliferative effect in both ER $^{+}$  and ER $^{-}$  cell lines and below 10  $\mu$ M genistein has a proliferative effect (Wang and Kurzer, 1997; Zava and Duwe, 1997). It is believed that the chemopreventative effects of genistein are mediated through the estrogen receptor (Messina et al, 1994; Kurzer and Xu, 1997; Kuiper et al, 1998). Genistein has a low binding affinity for the estrogen receptor and may compete with estrogen for receptor sites without inducing the signals that estrogen would induce. Alternative mechanisms for the chemopreventative effects, such as the inhibition of tyrosine kinase activity, have also been proposed (Messina et al, 1994; Kurzer and Xu, 1997; Kuiper et al, 1998).

Genistein has also been shown to have effects that are not mediated through the estrogen receptor. *In vitro*, genistein is able to stimulate the synthesis of hormone binding globulin, which can possibly lead to a decrease in circulating hormones *in vivo* (Adlercreutz et al., 1992). In the ovaries, the aromatase enzyme activity, which converts androgens to estrogens, is inhibited by genistein and other phytoestrogens, equol and biochanin A (Wang et al., 1994; Pelissero et al., 1996; Chen et al., 1997). The 17 $\beta$ -hydroxysteroid oxidoreductase Type I, which converts estrone to estradiol, was also shown to be blocked by genistein (Franke and Custer, 1994). Furthermore, genistein also inhibits tyrosine protein kinase, which phosphorylates tyrosine residues such as growth factors involved in proliferation (Piontek et al., 1993; Boutin, 1994), and DNA

topoisomerases I and II, which are essential for DNA replication (Okura et al., 1988; Kurzer and Xia, 1997).

### **Estrogen and Estrogenic Chemicals and the Immune System**

**Gender Differences:** Gender differences in B cell and T cell mediated immunity are well established (Grossman, 1984; Ansar Ahmed et al., 1985a; Olsen and Kovacs, 1996). Females typically have stronger primary and secondary B-cell-mediated responses to a variety of antigens, and higher levels of serum immunoglobulins (Butterworth et al., 1967; Ansar Ahmed et al., 1985a; Olsen and Kovacs, 1996). Furthermore, women have increased CD4<sup>+</sup> T cell numbers in the peripheral blood (Amadori et al., 1995; Olsen and Kovacs, 1996). In fact, a decrease in CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio in the peripheral blood with an increase in the absolute number of lymphocytes and in the percentage of CD8<sup>+</sup> lymphocytes was demonstrated in women with estrogen deficiencies when compared with normal controls (Hoet al., 1991). Differences in cytokine production have also been noted between genders. Interferon- (IFN-)  $\gamma$  and interleukin- (IL-) 4 production is higher in lymph node cells from female mice than males mice (Sarvetnick and Fox, 1990; Araneo et al., 1991). In normal mice, IL-4 production is increased in splenocytes during pregnancy (Dudley et al., 1993). IL-1 is also elevated in females compared to males in peritoneal macrophages in rats (Hu et al., 1988) and mononuclear cells and urinary secretions in humans (Lynch et al., 1994). Furthermore, females exhibit increased resistance to a variety of bacterial, viral, and parasitic infections (Willoughby and Watson, 1969; Rifkind, 1972; Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1990; Homo-Delarche et al., 1991).

**Estrogen Treatment:** Both *in vivo* and *in vitro* studies show that estrogen and estrogenic chemicals are able to modulate the immune system. Estrogen may have varied effects on B cells, depending upon the stage of cell development. Clonal expansion of B cell precursors in the bone marrow is suppressed by estrogen (Mazuzawa et al., 1994; Medina and Kincade, 1994; Smithson et al., 1995), while maturation of B cells is increased (Homo-Delarche et al., 1991; Home-Delarche and Durant, 1994). Furthermore, long-term estrogen treatment in normal mice induces a hyperactivity of splenic B cells that is characterized by increased production of autoantibodies to mouse erythrocytes (Ansar Ahmed et al., 1989), dsDNA (Verthelyi and Ansar Ahmed, 1994), phospholipids (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1997), and an increased output of autoantibodies from plasma cells (Verthelyi and Ansar Ahmed, 1998). Also, studies have shown that chlordecone, an estrogenic pesticide, increases antibody-forming cells in the spleen (Chetty et al.,

1993), and DDT, another estrogenic pesticide, suppresses anti-sheep red blood cell (RBC) antibodies in rats (Konner et al., 1998).

In T cell cultures, estrogen has been shown to increase the CD4<sup>+</sup> T cell subset, facilitate T cell maturation, and enhance T helper functions (Muller et al., 1995; Olsen and Kovacs, 1996). Conversely, both *in vivo* and *in vitro* studies have shown that estrogen inhibits T suppressor cell activity from splenic lymphocytes (Paavonen et al., 1981; Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990). The increase in the ratio of CD4<sup>+</sup>:CD8<sup>+</sup> T cells in females is also seen with estrogen treatment or castration in males, and the increase can be reversed by treatment with androgens (Olsen et al., 1991). Estrogen treatment also results in a shorter duration for graft rejection, which is thought to result from either an increase in T helper and inducer populations or a decrease in T suppressor and T cytotoxic cell activity (Olsen and Kovacs, 1996). In contrast, DDT treatment resulted in decreased delayed-type hypersensitivity in rats (Konner et al., 1998).

Both *in vivo* and *in vitro* studies have shown that estrogen also alters cytokine expression. T cell clones from multiple sclerosis patients exposed to 17- $\beta$ -estradiol exhibited a dose dependent increase in IFN- $\gamma$  and IL-10 production and a biphasic production of tumor necrosis factor (TNF) (Gilmore et al., 1997). High doses of 17- $\beta$ -estradiol caused a decrease in TNF production and low doses of 17- $\beta$ -estradiol caused an increase in TNF production. In these T cell clones, IL-4 and TGF- $\beta$  production was not altered by estradiol exposure (Gilmore et al., 1997). Murine studies have shown found that IL-2 and IFN- $\gamma$  production was increased in splenocytes from castrated C57BL/6 males stimulated with concanavalin A (Con A) when compared to intact males (Viselli et al., 1995). In contrast, a similar study in splenocytes from C3H mice stimulated with anti-CD3 did not find any changes in cytokine production (Araneo et al., 1991). *In vivo* administration of tamoxifen, an anti-estrogen, or anti-estradiol antibodies in Balb/c mice with experimental autoimmune encephalomyelitis exhibited a decrease in IFN- $\gamma$ , IL-2, and IL-4 production and an increase in TNF- $\alpha$  and IL-1 (Dayan et al., 1997). Furthermore, in an *in vitro* study, the increase in IFN- $\gamma$  production seen with estrogen treatment was due to an increase in the promoter activity in murine spleen cells (Fox et al., 1991). Another *in vitro* study has shown that zearalenone, an estrogenic metabolite produced by *Fusarium*, increases IL-2 and IL-5 levels in the EL4.IL-2 thymoma cell line (Marin et al., 1996). *In vitro*, 17- $\beta$ -estradiol has also been shown to enhance the lipopolysaccharide (LPS) induced IL-1 $\beta$  promoter activity and octylphenol has been shown to block this activation at high concentrations (Ruh et al., 1998)



Estrogen also inhibits natural killer (NK) cell activity (Seaman and Gindhart, 1979; Ansar Ahmed et al., 1989; Baral et al., 1995; Puzanov et al., 1996) and activation and recruitment of macrophages to inflammatory sites (Frazier-Jessen et al., 1996) in mice. Furthermore, genistein has been shown to increase the activity of human peripheral blood NK cells *in vitro* at concentrations less than 5  $\mu\text{mol/L}$  and inhibit the activity at concentrations greater than 5  $\mu\text{mol/L}$  (Zhang et al., 1999). Similarly, men that consumed fish contaminated with PCBs, endocrine disrupting industrial chemicals, had decreased NK cell activity compared to those who did not consume fish (Svensson et al., 1994).

*In vitro*, octylphenol, an estrogenic metabolite of surfactants, affects splenocytes in what may or may not be an estrogenic mechanism. Octylphenol, unlike 17- $\beta$ -estradiol, has been shown to be toxic to splenocytes in a  $\text{Ca}^{2+}$ -dependent manner in culture (Nair-Menon et al., 1996). However, prior treatment of the splenocytes with 17- $\beta$ -estradiol is able to prevent the toxicity (Blake et al., 1997).

Immune changes have also been associated with exposure to estrogenic endocrine disrupting chemicals in Lake Apopka, the Great Lakes, and the Baltic Sea. Young alligators of Lake Apopka had decreased antibody responses and hypoplastic spleens and bone marrow (Schoeb et al., 1998). Furthermore, a decline in T-cell mediated immunity was noted in the caspian tern chicks and herring gulls of the Great Lakes (Grasman, 1995), and a suppression of delayed-type hypersensitivity and antibody response in harbor seals that fed on herring was related to contamination in the Baltic Sea (Ross et al., 1995). Environmental contamination with DDT and PCBs has been associated with immune suppression in many studies also. High levels of DDT and PCBs were associated with a decrease in the proliferative response of peripheral blood lymphocytes to T cell mitogens in bottlenose dolphins (Lahvis et al., 1995). Furthermore, the immunosuppression induced by DDT and PCBs exposure is suspected in the large-scale mortality noted in bottlenose dolphins due to increased infection (Lahvis et al., 1995). In another study on Beluga whales that were exposed to p,p' DDT or PCBs, splenocytes and peripheral blood leukocytes showed a decreased proliferative response to phytohaemagglutinin (PHA) (De Guise et al., 1998). Similar to the immune suppression seen in the wildlife from endocrine disrupting chemical exposure, mice exposed to 17- $\beta$ -estradiol and DES exhibited suppressed host resistance to a variety of pathogens, including *Listeria monocytogenes* (Pung et al., 1984; Pung et al., 1985), *Toxoplasma gondii* (Pung and Luster, 1986), and *Trichinella spiralis* (Luebke et al., 1984).

## **Estrogen and Estrogenic Chemicals and the Thymus**

**Thymus:** The thymus is the primary lymphoid organ in which T cell development takes place. It is a bilobed organ that is further divided into lobules. Each lobule has an outer cortex with an inner medullary region. The cortex contains immature proliferating thymocytes with epithelial nurse cells in the outer cortex and cortical epithelial cells forming the epithelial network. The medulla contains the mature thymocytes with the medullary epithelial cells organized into clusters. Macrophages and interdigitating dendritic cells can mainly be found in the corticomedullary junction.

The thymus is the site of T cell differentiation and selection. The immature CD4<sup>-</sup>CD8<sup>-</sup>T cell receptor (TCR) - pro-thymocytes migrate from the bone marrow to the cortex of the thymus. In the cortex, after many intermediary steps, thymocytes become CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>low</sup> and they undergo positive selection. During positive selection, those thymocytes with an affinity for the self-major histocompatibility complex (MHC) do not undergo programmed cell death (PCD), or apoptosis, whereas those thymocytes that are not able to engage or recognize the self-MHC die by apoptosis. Therefore, in theory, all the T cells that survive positive selection are self-MHC restricted. The surviving thymocytes then differentiate in either CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>high</sup> or CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>high</sup> T cells. The thymocytes then undergo negative selection in order to eliminate any T cells that will react to self-antigens. In negative selection, those T cells that bind with a high affinity to MHC molecules on macrophages, dendritic cells, or epithelial cells presenting self-antigens or to MHC molecules without antigen undergo apoptosis. In contrast those T cells with low or no affinity to MHC molecules with or without antigen survive. Therefore, those T cells that survive the selection process should recognize only foreign antigens presented on self-MHC molecules, leading to self-tolerance. Approximately 97% of the thymocytes will die by apoptosis as they mature and undergo positive and negative selection within the thymus. The mature T cells then migrate through the medulla and exit the thymus into circulation.

**Estrogenic Effects:** It is well established that estrogen is able to induce atrophy of the thymus. In women, the peak serum estradiol concentration of the menstrual cycle inversely correlates with lymphocyte cell numbers in the peripheral blood (Mathur et al., 1979, Danel et al., 1983). Furthermore, atrophy of the thymus is seen during pregnancy in mice, a time of high serum estrogen concentrations, and is characterized by a loss of cortical cells (Phuc et al., 1991). Castration or estrogen treatment of male rodents has also been shown to induce thymic atrophy (Grossman, 1984). Histological examination showed a loss of thymocytes, atrophy of the lobules, and an increase in the fat content of the

thymus (Grossman, 1984). Further studies found that the atrophy is due to a decrease in immature, CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in the cortex, leading to atrophy of the cortex (Scarpanti et al., 1989, Silverstone et al., 1994). Also, hypoplastic thymuses have been noted in the same young alligators from Lake Apopka in which feminization of the reproductive tracts was found (Schoeb et al., 1998). The structure of the thymus is important in the differentiation and selection of T cells. Critical steps in both differentiation and selection occur in the cortex, prior to the migration of thymocytes into the medulla. The loss of the structure of the cortex could lead to alterations in differentiation and selection of T cells and ultimately to a breakdown in tolerance and autoimmunity.

**Apoptosis:** PCD is an essential process in both positive and negative selection in the thymus. Apoptosis is a term that describes morphological changes in cells undergoing PCD (Cohen, 1991; Cohen and Richard, 1992). With apoptosis there is cell shrinkage, flipping of the phosphatidyl serines from the inner to the outer membrane, condensation and systematic cleavage of the DNA, and apoptotic body formation, which are then engulfed by surrounding cells, and thus prevention of an inflammatory response (Wyllie, 1993). In contrast, necrosis is characterized by swelling of the cell and release of internal chemotactic cell contents, which leads to an inflammatory response. Apoptosis is an essential part of the development of self-tolerance in the positive and negative selection process. Any defect in the signaling of apoptosis during selection can lead to a breakdown in self-tolerance. A break down in self-tolerance may result in autoimmunity and ultimately lead to autoimmune disease. Estrogen treatment in many species has been shown to increase the incidence of autoimmune disease; however, as of yet, there is no evidence that estrogens directly induce apoptosis of thymocytes. Furthermore, studies have shown that DES, a synthetic estrogen, does not directly induce apoptosis of thymocytes *in vitro* (Donner et al, 1999).

### **Estrogen and Estrogenic Chemicals and Autoimmune Disease**

**Gender Differences:** Autoimmune disease afflicts 1 in 31 Americans (Jacobson et al., 1997). Genetics, viral infections, stress, and sex hormones have all been implicated in autoimmune disease, however the precise contribution of each of these factors is still unclear (Talal, 1981). However, it is well established that women are more susceptible to autoimmune disease than men (Ansar Ahmed and Talal, 1989, 1993, 1998; Ansar Ahmed et al., 1999; Olsen and Kovacs, 1996; Sullivan, 1997b; Wilder, 1998; Lahita, 1999). One epidemiologic study of 24 autoimmune diseases found that the majority of the 9 million people in the USA that were afflicted were women (Jacobson et al., 1997). The female to male ratio for systemic lupus erythematosus (SLE) and Sjogren's Syndrome is 9:1, thyroid

disease 25:1, autoimmune diabetes mellitus 5:1, scleroderma 3-4:1, autoimmune adrenal disease 2-3:1, rheumatoid arthritis 2-4:1 (Ansar Ahmed et al., 1985a; Homo-delarche et al., 1991), and multiple sclerosis 2-2.5:1 (Duquette et al., 1992).

***Influences of Estrogen:*** Some believe that the increased incidence of autoimmune disease in women is due to X-chromosomal activation (Stewart, 1998), but the majority of evidence points to modulation of the disease by sex hormones. Women with SLE have been shown to have lower androgen levels than age-matched normal control (Jungers et al., 1982a; Lahita et al., 1987; Apelgren et al., 1996), and abnormal sex hormone metabolism has been noted in women with SLE and Klinefelter's syndromes (Lahita et al., 1979, 1981; Talal, 1979; Ansar Ahmed et al., 1985a; Ben-Chetrit 1994). Women with SLE have an increase in 16 $\beta$ -hydroxylated estrogen metabolites, which presumably have more potent estrogenic activity (Lahita et al., 1981). Furthermore, the course of autoimmune disorders has been shown to be modulated by alterations in estrogen levels in the body, such as during pregnancy, oral contraceptive use, and menopause (Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1988, 1990, 1993, 1998; Lahita, 1990)). Three healthy women that received estrogen treatment for ovulation induction for primary and secondary infertility developed SLE within 1-3 months (Ben-Chetrit and Ben-Chetrit, 1994). A link between prenatal DES exposure and autoimmune disease has also been suggested by some (Noller et al., 1988; Wingard and Turiel, 1988), although another preliminary study did not find this connection (Baird et al., 1996). Finally, fluctuating estrogen levels during pregnancy and estrogen therapy have been shown to exacerbate the progression of certain autoimmune disorders, such as SLE (Jungers et al., 1982b), autoimmune thyroiditis (Amino, 1977a), Grave's disease (Amino, 1977b; Watanobe and Kawabe, 1996)

***Animal Models:*** The increased incidence of autoimmune diseases in females is also seen in the animal models for the human autoimmune disorders (Ansar Ahmed et al., 1985a, 1999; Ansar Ahmed and Talal, 1989; Hahn, 1999; Theofilopoulos and Kono, 1999). In the (SWRxSJL)<sub>F1</sub>, (SNF<sub>1</sub>) and the (NZBxNZW)<sub>F1</sub>, (B/W), mouse models for SLE, the females have an earlier onset of the disease and die at an earlier age than the males (Hahn, 1999). Females in a mouse model for arthritis have an increased ability to degrade implanted cartilage, with an increase in IL-1 in the granulomata from the females compared to the males (Da Silva et al., 1993). The rat model for autoimmune thyroiditis has a 3:1 ratio for incidence of the disease in females compared to males (Ansar Ahmed et al., 1985a). The non-obese diabetes (NOD) mouse model has an increase in the incidence of spontaneous diabetes in females compared to males (Fitzpatrick et al., 1991). The incidence of hemolytic anemia is higher in female

mice compared to males; the polyarthritis ratio of female to male incidence is 6:1 in LEW/N rats and 3:1 in LEW/N<sub>x</sub>F344/N<sub>F1</sub> rats; and for dogs the female to male ratio of incidence of autoimmune thrombocytopenia is 2:1 (Ansar Ahmed et al., 1985).

These animal models have allowed a significant amount of research on the ability of sex hormones to modulate the onset and progression of autoimmune diseases. In the thymectomized-irradiated rat model for thyroiditis, both the onset and the severity of the disease is modulated by sex hormones (Ansar Ahmed and Penhale, 1980). Testosterone has been shown to be beneficial in both the prevention and the cure of thyroiditis in the rat model of autoimmune thyroiditis (Ansar Ahmed et al., 1986). Furthermore, in the SJL mouse model for experimental autoimmune encephalomyelitis, which is induced by transfer of myelin basic protein specific T cells, dihydrotestosterone was able to diminish the severity of the disease. An enhanced production of IL-10 by antigen specific T lymphocytes was noted in the animals with diminished severity (Dalal et al., 1997). The susceptibility of male NOD to spontaneous diabetes is increased with orchietomy, and combined orchietomy + thymectomy further increases the incidence of the disease. It is theorized that the androgens may be acting through the gonadal-thymic axis (Fitzpatrick et al., 1991). Modulation of the onset and progression of SLE by sex hormones has also been found in the mouse models. Male B/W mice that have a depletion of male hormones or an increase in estrogen through anti-androgens, orchietomy, or estrogen treatment exhibit an accelerated onset of the disease (Roubininan et al., 1978; Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1988, 1990, 1993, 1998; Walker et al., 1994). In contrast, female B/W mice treated with androgens or anti-estrogens are more resistant to the disease and have a delayed onset (Roubininan et al., 1978; Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1990, 1993, 1988, 1998;). In the SNF<sub>1</sub> model of SLE, male mice exhibit increased susceptibility to disease with estrogen treatment (Gavalchin et al., 1998). The susceptibility with estrogen treatment seemed to be mediated by an increase in CD4<sup>+</sup> T cells. Increased CD4<sup>+</sup> cells were found in the thymus, spleen, and kidneys (Gavalchin et al., 1998). Furthermore, disease in both the MRL/lpr model for SLE, which is based on a dysfunctional Fas receptor, and the immunized model for SLE, which is immunized with the 16/6 idiotypic bearing anti-DNA antibodies, is promoted by estrogen treatment (Carlsten et al., 1990; Dayan et al., 1997). Sjogren's syndrome, which is noted by autoimmune infiltrates in the lacrimal and salivary glands, is reduced by testosterone treatment in the MRL/lpr and B/W models (Ariga et al., 1989, Sullivan, 1997a).

**(NZBxNZW)<sub>F1</sub> Model for SLE, (B/W):** The B/W murine model for SLE is believed by many to be the model that most closely resembles human SLE (Ansar Ahmed et al, 1985; Hahn, 1999). Furthermore, it is well established that the B/W model is sensitive to sex hormone treatment, especially for modulation of the onset and the progression of the disease (Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1993, 1998; Walker, 1999). The disease is enhanced/accelerated by the administration of estrogens and the depletion of male hormone levels (by orchiectomy and/or anti-androgen treatment). Conversely, the disease can be suppressed by the administration of androgens or anti-estrogens. It is for this reason, the B/W model was chosen to investigate the effects of EDC in these studies. Both the New Zealand black (NZB) and the New Zealand white (NZW) parents contribute genetically to the immune abnormalities that cause the disease in the F1 generation. NZW mice have a slightly shorter life span with intermittent autoantibody production throughout, leading to development of nephritis late in life in some mice (Hahn and Shulman, 1969; Kelley and Winkelstein, 1980). NZW mice also have a deletion in the  $\beta$  chain of the T cell receptor, but this has not been shown to contribute to the disease (Hahn, 1999). The NZB mice show B cell hyperactivity, with an abnormally high secretion of immunoglobulins by one month of age (Steinberg et al., 1981; Yoshida et al., 1990). The genes for high immunoglobulin production are passed on to the B/W mice; however, the B/W mice exhibit a strong T cell dependence of the secretion, which is probably responsible for the switch from IgM to IgG anti-double stranded DNA (dsDNA) antibodies (Steward and Hay, 1976; Papoian et al., 1977). The expression of the estrogen receptor (presumably ER $\alpha$ ) is comparable between the NZB, NZW, and B/W mice in the uterus, thymus, and the spleen. However, in the liver, the expression of the estrogen receptor is two times higher in the NZW and B/W compared to the NZB (Athreya et al, 1989).

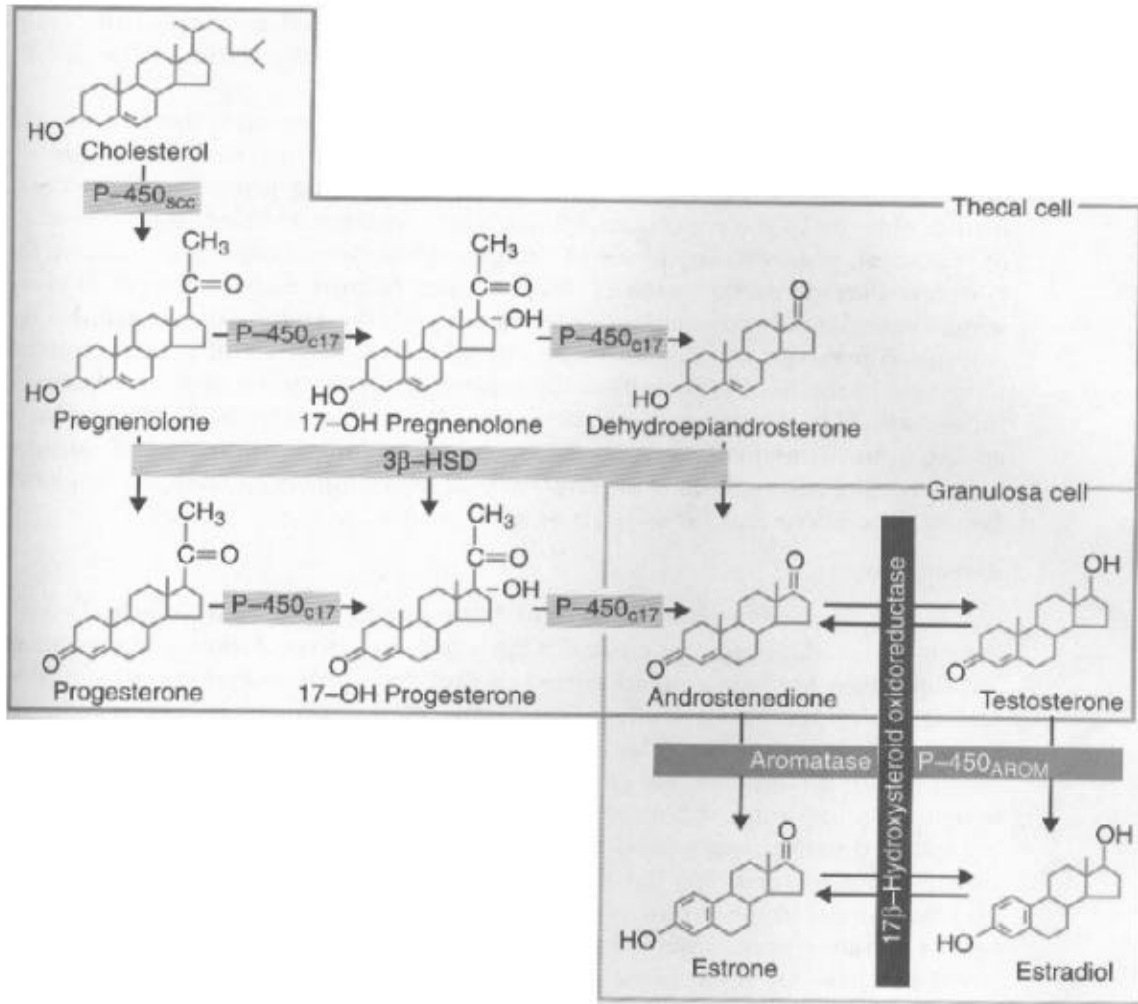
The B/W mice die of glomerulonephritis due largely to deposition and complement fixation of anti-dsDNA autoantibodies in the kidneys (Andrews et al., 1978; Steinberg et al., 1981; Theofilopoulos and Dixon, 1981; Yoshida et al., 1990). Females die at an average of 280 days, whereas, males die at an average of 439 days. Anti-nuclear antibodies (particularly IgM), including antibodies to dsDNA, single stranded DNA (ssDNA), dsRNA, tRNA, histones, and polynucleotides, appear at 2-3 months in females (Munns et al., 1982; Park et al., 1983; Ballard and Voss, 1985; Brick et al., 1990; Fisher et al., 1988; Ohnishi et al., 1994). IgM anti-dsDNA antibodies rise in females between 3-5 months in age, and an isotype switch occurs and IgG dsDNA antibodies appear between 5-7 months (Steward and Hay, 1976; Papoian et al., 1977; Andrews et al., 1978). The large IgG dsDNA antibody production is abrogated by the removal to CD4+ T

cells (Wofsy and Seaman 1985, 1987). Proteinuria is seen at 5-7 months in the female, shortly after the isotype switch because the IgG isotypes fix complement well, especially the IgG2a and IgG2b (Andrews et al., 1978). Males also develop anti-nuclear antibodies, and the IgM to IgG isotype switch occurs at ~12 months of age. Males generally die between 12-15 months from chronic nephritis (Andrews et al., 1978; Ohgaki et al., 1989). Histological changes, apart from those in the kidneys, occur in other tissue. Lymphocyte infiltration is seen in the salivary glands, along with a mild inflammation around the bile duct in the liver. Furthermore, pancarditis, vasculitis, myocardial infarcts, and deposits of DNA and anti-DNA antibodies in the dermo-epidermal junction are seen (Andrews et al., 1978; Jabs and Prendergast, 1994; Chandrasekar et al., 1995).

B cell and T cell abnormalities have been noted in B/W mice that may contribute to the disease. Mainly, the hyperactivity of B cells, which leads to an abnormally high level of antibody production at an early age. The antibody production is dependent upon CD4<sup>+</sup> T cells (Wofsy and Seaman, 1985, 1987), and as the mice age the level of CD4<sup>+</sup> T cells increase 5-fold (Rozzo et al., 1994). Thymic epithelial and cortical atrophy is seen by 6 months of age. Furthermore, IL-1 secretion from macrophages is decreased in B/W mice (Hartwell et al., 1994; Hartwell et al., 1995), and CD8<sup>+</sup> cells are decreased in numbers and possibly suppressor activity (Ito et al., 1992).

As can be seen from a review of the literature, estrogen and many estrogenic chemicals are able to modulate, not only the reproductive system, but also many aspects of the immune system. Octylphenol and genistein are two endocrine disrupting chemicals in our environment that are able to mimic estrogenic effects on the reproductive tract and have been shown to effect immune cells *in vitro*. Therefore, it is necessary to determine the effects of *in vivo* exposure to octylphenol or genistein on the immune system.

## Estrogen Production in the Ovaries



**Fig. 1.1:** Diagram of estrogen production in the thecal and granulosa cells of the ovaries. (Kettyl and Arky, Endocrine Pathophysiology, p. 235)



## Chapter 2

### **Preliminary studies to determine the effects of short-term oral dosing of octylphenol and genistein on the thymus, spleen, and lymph nodes of C57BL/6 or B/W mice**

#### **a.) Introduction**

There is an extensive body of literature that exhibits estrogenic modulation of the immune system. It includes: the identification of the estrogen receptor on immune cells, gender differences in immune responses, and modulation of the progression of autoimmune disease by estrogen (Grossman, 1984; Ansar Ahmed et al., 1985a; Olsen and Kovacs, 1996). Furthermore, a new class of compounds, EDC, has emerged and is continually growing. Many EDCs, which are able to mimic estrogen in the body, have been found in the environment. octylphenol and genistein are two EDCs that have been shown to mimic estrogen both *in vitro* and *in vivo* (Arts et al., 1989; Whitten et al., 1993; White et al., 1994; Levy et al., 1995; Blake and Boockfor, 1997; Coldham et al., 1997). Furthermore, both chemicals possess non-estrogenic properties that may also enable them to modulate the immune system. Many studies have reported the ability of genistein and octylphenol to modulate the reproductive system; however few studies have investigated their ability to modulate the immune system. Preliminary *in vitro* studies in our laboratory examining the effects of octylphenol, genistein, methoxychlor, and zearalenone on primary isolations of lymphocytes from the thymus, spleen, and lymph nodes did not modulate the immune system. Therefore, this preliminary study investigates the *in vivo* effects of genistein and octylphenol on the immune systems of mice.

Male C57BL/6 and the B/W orchietomized mice were utilized in preliminary studies. The C57BL/6 strain is a 'normal' inbred strain and the B/W strain develops a lupus-like autoimmune disease. Both strains have been shown to be sensitive to estrogenic modulation of the immune system (Verthelyi and Ansar Ahmed, 1994, 1997, 1998; Walker, 1999). *In vivo* estrogen treatment has been shown to effect both B cell and T cell responses in orchietomized male C57BL/6 mice (Verthelyi and Ansar Ahmed, 1994, 1997, 1998; Hissong et al., unpublished data). Furthermore, estrogen has been shown to accelerate the onset of the lupus-like autoimmune disease in B/W males (Walker, 1999). Since both strains of mice have been shown to be sensitive to estrogen treatment, octylphenol was tested in the B/W strain and genistein was tested in the C57BL/6 strain. Furthermore, endogenous estrogens or androgens may interfere

in the effects of the estrogenically weaker EDCs. Therefore, B/W males were orchietomized in order to decrease the levels of endogenous androgens and estrogens and, hopefully, allow maximal effects of octylphenol.

### **b.) Specific Aim**

The aim of the preliminary study was to determine which, if any, of the lymphoid organs or cell populations of the immune system in the orchietomized or non-orchietomized male mouse are sensitive to the effects of octylphenol or genistein. The following morphological and functional properties were examined:

#### a.) Thymus

- ❖ Morphology: organ weight, cellularity, proportion of immature (CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup>) and mature (CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup>) thymocyte subpopulations
- ❖ Function: apoptosis and proliferation of unstimulated and stimulated thymocytes

#### b.) Spleen

- ❖ Morphology: organ weight, leukocyte cellularity, proportion of T cell and B cell subpopulations
- ❖ Function: proliferation, very early activation antigen expression (CD69), cytokine production, apoptosis, and entrance into the cell cycle upon stimulation

#### c.) Lymph Nodes

- ❖ Morphology: T cell and B cell subpopulations
- ❖ Function: proliferation, very early activation antigen expression (CD69), cytokine production, apoptosis, and entrance into the cell cycle upon stimulation

### **c.) Materials and Methods**

**Mice:** Eight to ten week old C57BL/6 males were purchased from Charles River (Wilmington, MA) and four to six-week old B/W males were purchased from the Jackson Laboratories (Bar Harbor, ME). All mice were housed two per cage, kept on a 14 hour light, 10 hour dark cycle, and fed a soy-depleted commercial pellet diet (Teklaad 7001 (NIH-31), Harlan Teklaad, Madison, WI) with water *ad libitum*. One week after arrival the B/W males were anesthetized with 0.625 – 1.25 mg of sodium pentobarbitol and orchietomized (Ansar Ahmed et al., 1989).

**Octylphenol treatment:** Five-month old orchietomized B/W males were fed 10 mg of octylphenol three times a week for three weeks. Previous studies have found this dose to be estrogenic in relation to the reproductive changes in male rats (Blake and Boockfor, 1997). Octylphenol (Sigma, St. Louis, MO) dissolved in ethanol at a stock concentration of 10 mg/20  $\mu$ l was utilized for treating B/W males. Twenty microliters of the stock was mixed with 10  $\mu$ l of autoclaved corn oil (ICN, Aurora, OH) and fed to the mice with a disposable tip on a micropipetter three times a week for three weeks. Control mice were fed 20  $\mu$ l of ethanol mixed with 10  $\mu$ l of corn oil with a micropipetter three times a week for three weeks. The corn oil was autoclaved to eliminate residual phytoestrogens and eliminate any contamination. Three days after the last treatment the mice were killed by cervical dislocation as approved by the Virginia Polytechnic Institute and State University Animal Care Committee.

**Genistein treatment:** Five-month old C57BL/6 males were fed 0.6 mg of genistein everyday for three weeks. The dose of genistein is based on studies in mice that have examined its effect on luteinizing hormone release and DNA adduct formation in bone marrow (Hughes et al., 1991; Giri and Lu, 1995). A 1M (270.2 g/L) stock of genistein (Calbiochem, La Jolla, CA) dissolved in dimethylsulfoxide (DMSO) (Fisher Scientific, Pittsburg, PA) was aliquoted and kept  $-20^{\circ}\text{C}$  and utilized for these studies. A 1:7 dilution of the genistein stock solution was made in autoclaved corn oil everyday and the 20  $\mu$ l was fed to each mouse in the genistein treatment group with a disposable tip on a micropipetter. The control mice were fed 20  $\mu$ l of a 1:7 dilution of DMSO in corn oil with a micropipetter. The day after the last treatment the mice were killed by cervical dislocation.

**Tissue collection and processing:** The mice were weighed upon sacrifice. The spleen, thymus, and lymph nodes were removed under sterile conditions, and the spleen and thymus were weighed sterilely. The body weights taken prior to sacrifice were utilized in the % body weights ratios reported. The spleen, thymus, and lymph nodes were gently teased on a sterile 60-mesh steel screen as described in earlier studies (Ansar Ahmed et al., 1989). All cells were washed with RPMI media (CellGro, Mediatech, Herndon, VA). The erythrocytes were removed from the splenic cell suspension by lysis in ACK lysis buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 1.0 mM  $\text{KHCO}_3$ , 0.1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.4) as previously described (Ansar Ahmed et al., 1994). All cells were then washed in complete RPMI media (RPMI media supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT), 2mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/ml penicillin (Mediatech), and non-essential amino acids (Fisher)). The lymphocytes

in the thymus and leukocytes in the spleen and lymph nodes were then counted and the viability was assessed by trypan blue exclusion. The number of lymphocytes and leukocytes isolated from each organ was noted, and all cells were adjusted to a density of  $5 \times 10^6$  cells/ml in complete RPMI and kept at  $4^\circ\text{C}$  until utilized in assays.

**Flow cytometric analysis of cell markers:** Cells from the thymus, spleen, and lymph nodes were stained with appropriate monoclonal antibodies and analyzed by flow cytometry as previously reported (Ansar Ahmed et al., 1994). Briefly,  $100 \mu\text{l}$  of cells at  $5 \times 10^6/\text{ml}$  were plated into 96-well round bottom tissue culture plates (Corning, NY). One hundred microliters of diluted fluorochrome-conjugated monoclonal antibody in incomplete media, or the appropriate species and isotype matched control antibody in incomplete RPMI media were added to each well. The cells were incubated with the antibodies in the dark at  $4^\circ\text{C}$  for 30 minutes. The cells were then washed and analyzed for fluorescence on an EPICS XL-MXL flow cytometer (Coulter, Hialeah, FL). The following antibodies were utilized in these studies: fluorescein isothiocyanate (FITC)-conjugated anti-CD8a (53-6.7), anti-CD90 (Thy1.2, G7), anti-CD11b (Mac-1, M1/70), and a rat IgG<sub>2a</sub> isotype control (R35-95) or phycoerythrin (PE)-conjugated anti-CD4 (RM4-5), anti-CD19 (1D3), and rat IgG<sub>2a</sub> isotype control (R35-95). All monoclonal antibodies were purchased from Pharmigen (San Diego, CA).

**Stimulation and expression of Very Early Activation Antigen (CD69):** One hundred microliters of cells from the thymus, spleen, and lymph nodes at a density of  $5 \times 10^6$  cells/ml were added to 96-well round bottom plates. One hundred microliters of complete RPMI media containing 10 ng/ml of phorbol myristate acetate (Sigma) and  $0.5 \mu\text{g}/\text{ml}$  of ionomycin (Calbiochem) (PMA+I) were added to each well. The cells were incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . After 6 hours, the plate was centrifuged at  $300 \times g$  for 6 minutes to pellet the cells and the supernatants were removed. The cells were resuspended in  $100 \mu\text{l}$  of incomplete RPMI media and stained as in the previous section for cell surface markers. The cells were stained with either PE-conjugated anti-CD69 (H1.2F3) (Pharmigen) or PE-conjugated hamster IgG isotype control (A19-3) (Pharmigen) and analyzed by the flow cytometer for fluorescence.

**Stimulation and cell cycle analysis:** One hundred microliters of cells from the thymus, spleen, and lymph nodes at a density of  $5 \times 10^6$  cells/ml were added to 96-well round bottom plates. Thymocytes were stimulated with  $100 \mu\text{l}$  of complete RPMI media containing  $15 \mu\text{g}/\text{ml}$  of anti-CD3 antibodies (YCD3, purified in our lab), or complete RPMI media containing 10 ng/ml of phorbol

myristate acetate and 0.5  $\mu\text{g}/\text{ml}$  of ionomycin (PMA+I). Thymocyte cultures that received 100  $\mu\text{l}$  of complete media only served as base-line controls. Leukocytes from the spleen and lymph nodes were exposed to 100  $\mu\text{l}$  of complete media, complete media containing 10  $\mu\text{g}/\text{ml}$  concanavalin A (Con A, ICN), complete RPMI media containing 10  $\mu\text{g}/\text{ml}$  lipopolysaccharide (LPS, Sigma), or complete media containing 10 ng/ml of phorbol myristate acetate and 0.5  $\mu\text{g}/\text{ml}$  of ionomycin. Plates were incubated at 37°C, 5% CO<sub>2</sub> for 8, 12, 24, or 48 hours. After incubation, the cells were pelleted at 200 X g for 5 minutes and resuspended in Vindelov's propidium iodide solution followed by the addition of sodium azide buffer as described previously (Robinson et al., 1993; Zhi-June et al., 1998). Samples were incubated overnight at 4°C followed by flow cytometric analysis using DNA cell cycle analysis software. The cells are divided into the sub G<sub>0</sub> (or apoptotic), G<sub>1</sub>/G<sub>0</sub>, or S and G<sub>2</sub>/M phases of the cell cycle. The data are presented as the change from the media baseline of the stimulated cells in the proportion of cells in each phase.

**Alamar Blue proliferation assay:** Lymphocytes from the thymus and leukocytes from the spleen and lymph nodes were stimulated as described above for cell cycle analysis. Proliferation was determined utilizing the non-radioactive Alamar Blue assay. The Alamar Blue dye (Accumed International, Westlake, OH) contains a redox indicator that changes from a blue to a red color and from non-fluorescent to fluorescent state in the microenvironment of cellular proliferation. The extent of color change or the intensity of fluorescence is a reflection of the cellular proliferation. Studies in our laboratory have shown that the Alamar Blue assay is an indirect method of detecting cellular proliferation that is comparable to the <sup>3</sup>H-thymidine incorporation method (Ansar Ahmed et al., 1994, Zhi-Jun et al., 1998). Briefly, 20  $\mu\text{l}$  of Alamar Blue dye were added to each well at the same time the cells and stimulants were added. The cells were incubated with stimulants and Alamar Blue dye for 48 hours at 37°C, 5% CO<sub>2</sub>. At 8, 12, 24, and 48 hours of incubation, the fluorescence of each well was determined utilizing the CytoFluor II, fluorescence multiwell plate reader (PerSpectives Biosystems, Framington, MA) at excitation 530 nm, emission 590 nm, and a gain of 35. The data are presented as the change from the media baseline of the stimulated cells in the fluorescence readings.

**IL-2 and IFN-g ELISA:** Leukocytes isolated from the spleen and lymph nodes were stimulated as described above for cell cycle analysis. Cells were incubated for 24 and 48 hours at 37°C, 5% CO<sub>2</sub>, and subsequently, cells were pelleted by centrifuging at 300 X g for 6 minutes. The supernatants were removed and frozen for use in an enzyme-linked immunosorbent assay (ELISA)

to determine cytokine levels. A sandwich ELISA was utilized to determine the levels of IL-2, and IFN- $\gamma$ . High-binding, flat bottom 96-well Nunc-immunoplates with MaxiSorb surface (Nunc, Denmark) were coated with 50  $\mu$ l of rat anti-mouse IL-2 (JES6-1A12, Pharmigen) or rat anti-mouse IFN- $\gamma$  antibodies (R4-6A2, purified in our lab) at 2  $\mu$ g/ml in PBS. The plates were incubated overnight at 25-27°C and then washed three times with wash buffer (50 mM Tris with 0.2% Tween 20, pH 7.0-7.4). The plates were then blocked with 200  $\mu$ l of 2% bovine serum albumin (BSA) in PBS per well and incubated for one hour. Supernatants were diluted in complete media and 100  $\mu$ l/well were added to each plate after three washes. Serial dilutions of known standards were also added to each plate at 100  $\mu$ l/well. The samples and standards were incubated for 3 hours. After incubation, the plates were washed three times and anti-IL-2 (TES6-5H4, Pharmigen) or anti-IFN- $\gamma$  (XMG1.2, Pharmigen) biotinylated detecting antibody were diluted 1:2000 and added at 100  $\mu$ l/well to each plate. Plates were incubated for 1 hour and washed again three times. Horse-radish peroxidase-conjugated streptavidin (Vector Labs, Burlingame, CA) was diluted 1:4000 and 100  $\mu$ l was added/well to each plate. The plates were incubated covered for 30 minutes and then washed three times. The TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was then added at 100  $\mu$ l/well and incubated in the dark for 30 minutes. One hundred microliters of a 0.18 M H<sub>2</sub>SO<sub>4</sub> solution were added to each well to stop the reaction and the absorbance of the plates was read at 450 nm on the Molecular Devices kinetic plate reader. The concentrations of cytokines per well of the ELISA plate were determined utilizing known standards on each plate and the concentration of cytokines per sample of supernatants was determined by the concentration per well and the dilution utilized for the ELISA.

**Statistics:** The MIXED procedure of SAS (SAS ver. 6.12, SAS Institute, Cary, NC) was used to perform a mixed effects, repeated measures analysis of variance (ANOVA) on the cell cycle and proliferation data. A first order autoregressive model was used to model covariance among repeated measurements from a mouse. Cell cycle and proliferation data were analyzed as the change from the media baseline. All other data were analyzed utilizing the GLM procedure of SAS to perform a one-way ANOVA. For all data,  $p < 0.05$  was considered significant.

#### **d.) Results**

**Effect of octylphenol treatment on the morphology of the thymus in orchietomized B/W males:** Octylphenol treatment altered the morphology of the thymus in orchietomized B/W males. The number of thymocytes recovered

from the thymus of octylphenol treated mice was significantly less than the number recovered from control mice (Fig 2.1a). However, a comparable decrease in the percent body weight of the thymus was not seen (Fig 2.1b). Furthermore, no change in the proportion of immature (CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes or presumably mature (CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup>) thymocytes was noted (Fig 2.2).

**Effect of octylphenol treatment on the function of thymocytes in orchietomized B/W males:** The effect of octylphenol treatment on the proliferation or level of apoptosis of stimulated thymocytes was not statistically significant (Fig 2.3, 2.4). The proliferation of thymocytes induced by anti-CD3 antibodies or PMA+I at 8 and 24 hours of culture was not altered by octylphenol treatment (Fig 2.3). Furthermore, the percent of apoptotic cells at 8 and 24 hours of culture that were induced by stimulation with anti-CD3 antibodies and PMA+I was not statistically significant (Fig 2.4). However, a trend of increased induction of apoptosis compared to control was seen at 8 hours of culture by anti-CD3 stimulation and, to a lesser extent, PMA+I stimulation, but was gone by 24 hours (Fig 2.4).

**Effect of octylphenol treatment on the morphology of the spleen and lymph nodes in orchietomized B/W males:** No change in the number of splenic leukocytes or in the percent body weight of the spleen was noted in octylphenol treated mice (Fig 2.1a,b). Furthermore, octylphenol treatment neither altered the proportion of Thy1.2<sup>+</sup> T cells or CD19<sup>+</sup> B cells (Fig 2.5), nor altered the proportion of the T helper cells (CD4<sup>+</sup>) and T cytotoxic cells (CD8<sup>+</sup>) in the spleen or lymph nodes (Fig 2.5). Finally, the CD11b<sup>+</sup> developing granulocyte, monocyte and macrophage population in the spleen also was not affected by octylphenol treatment (Fig 2.5).

**Effect of octylphenol treatment on the function of lymphocytes from the spleen and lymph nodes in orchietomized B/W males:** The secretion of IFN- $\gamma$  by stimulated lymphocytes isolated from the spleen and lymph nodes was the only functional assay that appeared to be affected by octylphenol treatment. Splenic lymphocytes from octylphenol treated mice exhibited a trend of decreased secretion of IFN- $\gamma$  at 24 hours with Con A stimulation and at 48 hours with both Con A and PMA+I stimulation (Fig 2.6). Lymphocytes from the lymph nodes of octylphenol treated mice also exhibited a decrease in IFN- $\gamma$  secretion by 48 hours of culture with Con A and PMA+I stimulation, which was not seen at 24 hours of culture (Fig 2.7). However, at 48 hours the lymphocytes from the lymph nodes of one of the three control mice did have lower levels of IFN- $\gamma$  secretion than the three octylphenol treated mice (Fig 2.7). Upon stimulation with Con A or PMA+I, lymphocytes from the spleen and lymph nodes of octylphenol treated

and control mice secreted similar levels of IL-2 by 24 and 48 hours of culture (Fig 2.8). The expression of CD69 after 6 hours of stimulation with PMA+I did not change with *in vivo* octylphenol treatment (Fig 2.9). Furthermore, proliferation as assessed by Alamar blue and the levels of apoptosis and entrance into the cell cycle as assessed by propidium iodide staining were not altered in octylphenol treated mice. Leukocytes from the spleen and lymph nodes stimulated with a T cell stimulant (Con A), a B cell stimulant (LPS), or a pan stimulant (PMA+I) had similar changes from the media baseline in proliferation (Fig 2.10), apoptosis (sub Go – Fig 2.11, 2.12), and entrance into the cell cycle (S/G2/M - Fig 2.11, 2.12) at 24 and 48 hours whether they were from octylphenol treated mice or control mice.

**Effect of genistein treatment on the morphology of the thymus in C57BL/6 males:** *In vivo* feeding of genistein significantly decreased the percent body weight of both the thymus and the spleen in C57BL/6 males without a comparable decrease in lymphocyte numbers (Fig 2.13). The proportions of immature (CD4<sup>+</sup>CD8<sup>+</sup>) and mature (CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup>) thymocytes in the thymus was not altered by genistein treatment (Fig 2.14).

**Effect of genistein treatment on the function of thymocytes in C57BL/6 males:** *In vivo* treatment with genistein also did not alter the function of the thymocytes as assessed by proliferation and apoptosis. Anti-CD3 antibody and PMA+I induction of proliferation was not altered by genistein treatment as assessed by Alamar blue at 12 and 24 hours (Fig 2.15). Furthermore, anti-CD3 antibody and PMA+I induction of apoptosis was not altered by genistein treatment as assessed by propidium iodide staining at 12 and 24 hours of culture (Fig 2.16).

**Effect of genistein treatment on the morphology of the spleen and lymph nodes in C57BL/6 males:** *In vivo* feeding of genistein also significantly decreased the percent body weight of the spleen in C57BL/6 males without a comparable decrease in leukocyte numbers (Fig 2.13). The proportions of the T helper cells (CD4<sup>+</sup>) and T cytotoxic cells (CD8<sup>+</sup>) in the spleen and lymph nodes were not altered by genistein treatment (Fig 2.17). Furthermore, the proportion of Thy1.2<sup>+</sup> T cells and CD19<sup>+</sup> B cells in the spleen and lymph nodes and the proportion of the CD11b<sup>+</sup> developing granulocyte, monocyte and macrophage population in the spleen were not effected by genistein treatment (Fig 2.17).

**Effect of genistein treatment on the function of lymphocytes from the spleen and lymph nodes in C57BL/6 males:** *In vivo* treatment of genistein did not alter the function of the leukocytes isolated from the spleen and lymph nodes



as measured by CD69 expression, proliferation, activation-induced apoptosis, entry into the cell cycle, or cytokine expression. Stimulation of the leukocytes with PMA+I for 6 hours resulted in CD69 levels of expression that did not differ with genistein treatment (Fig 2.18). The induction of proliferation by a T cell stimulant (Con A), a B cell stimulant (LPS), or a pan stimulant (PMA+I) was not altered at 12, 24, or 48 hours by *in vivo* treatment with genistein (Fig 2.19). The induction of apoptosis (sub G<sub>0</sub>) and of entry into the cell cycle (S/G<sub>2</sub>/M) by Con A, LPS, and PMA+I did not differ between treatment groups at 24 or 48 hours for lymphocytes from the spleen (Fig 2.20) or at 48 hours for lymphocytes from the lymph nodes (Fig 2.21) as assessed by cell cycle analysis. Finally, the secretion of IL-2 and IFN- $\gamma$  induced by Con A and LPS stimulation was not altered by treatment with genistein (Fig 2.22).

### **e.) Discussion**

Both the genistein treatment in C57BL/6 males and the octylphenol treatment in B/W males, akin to estrogen, affected the morphology of the thymus. However, each treatment affected the thymus differently. The percent body weight of the thymus in C57BL/6 mice was decreased by treatment with genistein and the cellularity of the thymus in B/W mice was decreased by octylphenol treatment. The differential effects could be attributed to the different properties of the chemicals or the differences in the models in which they were tested. Neither genistein, nor octylphenol mimicked all the effect seen in previous studies with short-term 17 $\beta$ -estradiol treatment. Treatment with 17 $\beta$ -estradiol has been shown to result in thymic atrophy resulting from a decrease in the weight and cellularity of the thymus. The atrophy is due mainly to a loss of the immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in the cortex of the thymus (Scarpanti et al., 1989; Silverstone et al., 1994). Both genistein and octylphenol affected the thymus by causing either a decrease in the weight or cellularity of the thymus, but neither mimicked exactly the effects of 17 $\beta$ -estradiol treatment.

The decrease in the cellularity of the thymus in orchietomized B/W males could have resulted from many different mechanisms of action. The level of apoptosis at 8 hours of culture was not found to be significantly different in octylphenol treated mice from control mice, but a trend of increased apoptosis in octylphenol treated mice was noted. This could indicate an increase in apoptosis at early hours of culture that is not seen at later hours of culture, possibly due to the engulfment of apoptotic cells by macrophages and neighboring cells. It must also be noted that by 24 hours of culture most thymocytes are dead (Zhi-Jun et al., 1998; Donner et al., 1999). The increased apoptosis could not have targeted a certain cell population, because a change in the proportion of immature and

mature thymocytes was not noted. The decreased cellularity could also result from a decrease in the migration of precursor cells from the bone marrow or an increase in the migration of mature cell out of the thymus. In either case, a change in the migration rates would have to coincide with another alteration in the thymocyte population, because the proportion of immature and mature thymocytes was not altered by octylphenol treatment. Furthermore, a decrease in the percent body weight of the thymus was not noted. Therefore, the decrease in thymic lymphocytes may have coincided with an increase in the connective tissue, epithelial cells, or fat deposits in the thymus.

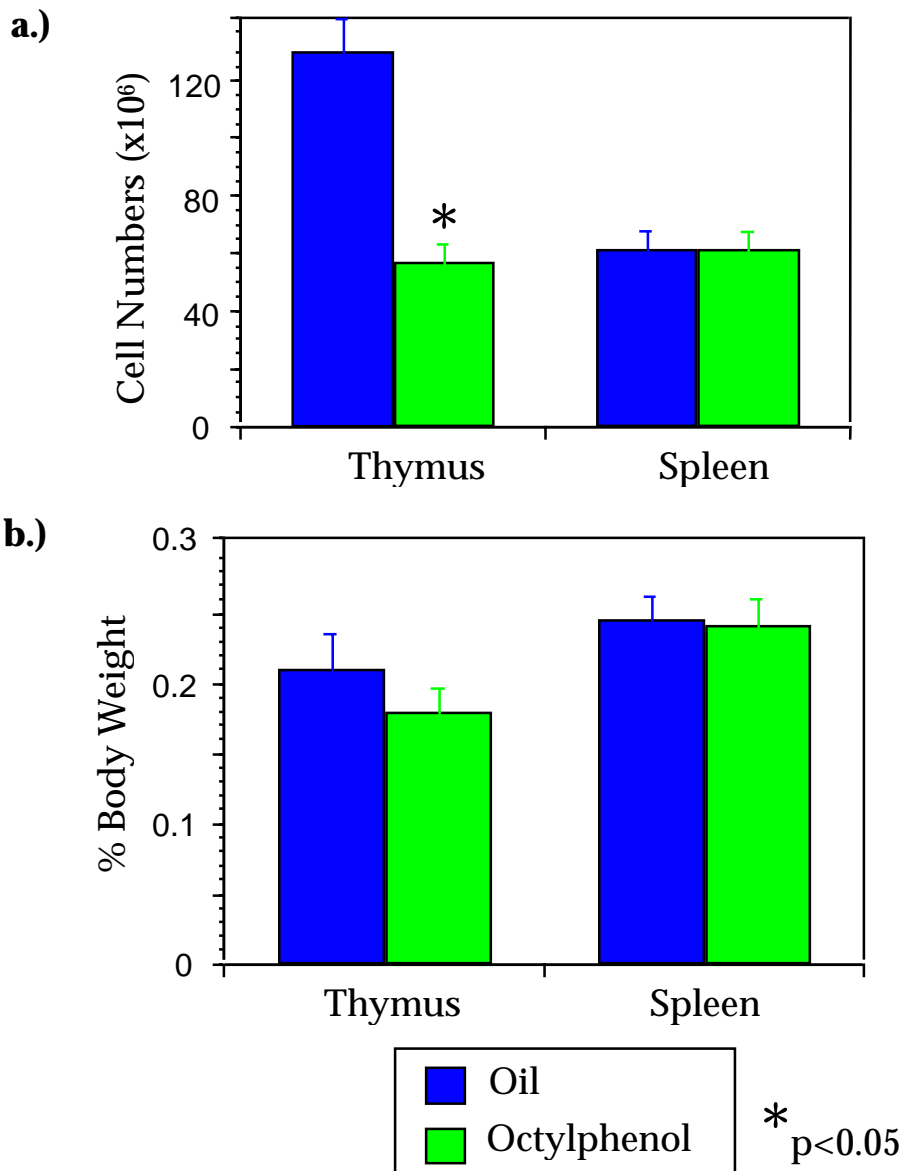
The decrease in the percent body weight of the thymus and spleen in C57BL/6 mice as a result of genistein treatment is more difficult to explain, because a comparable decrease in leukocyte cellularity was not seen. The decrease in the weight of the organs could have resulted from a decrease in the connective tissue, epithelial cells, or, in the case of the spleen, the red blood cells.

The morphology of the spleen and lymph nodes was not affected by octylphenol treatment in B/W males. However, octylphenol treatment may have affected the ability of the lymphocytes from the spleen and lymph nodes to secrete IFN- $\gamma$  upon Con A and PMA+I stimulation. IFN- $\gamma$  is an important cytokine in the stimulation of an immune response, especially a cytotoxic response. A decrease in the secretion of IFN- $\gamma$  could impair the immune systems ability to fight infection, especially intracellular infection. Further studies confirming a decrease in the ability of lymphocytes from secondary lymphoid organs to secrete IFN- $\gamma$  upon octylphenol treatment are necessary. Furthermore, it is necessary to determine the effect of decreased IFN- $\gamma$  secretion on the functioning of the immune system, such as the ability to fight infections that require a cytotoxic response. Also, it is necessary to determine the effects of octylphenol treatment in other models, such as females or reproductively immature male, and to determine the immunological effects of long-term octylphenol treatment.

In contrast, the function of the leukocytes isolated from the spleen and lymph nodes was not affected by genistein treatment in C57BL/6 males. The spleen and lymph nodes may not be sensitive to the estrogenic properties or non-estrogenic properties of genistein in the models in which we tested them. However, again, this was a short-term study. Long-term exposure to genistein may adversely affect the immune system. As with octylphenol, other models such as females or reproductively immature males may be more sensitive to the genistein, or a different route of exposure may be more effective.

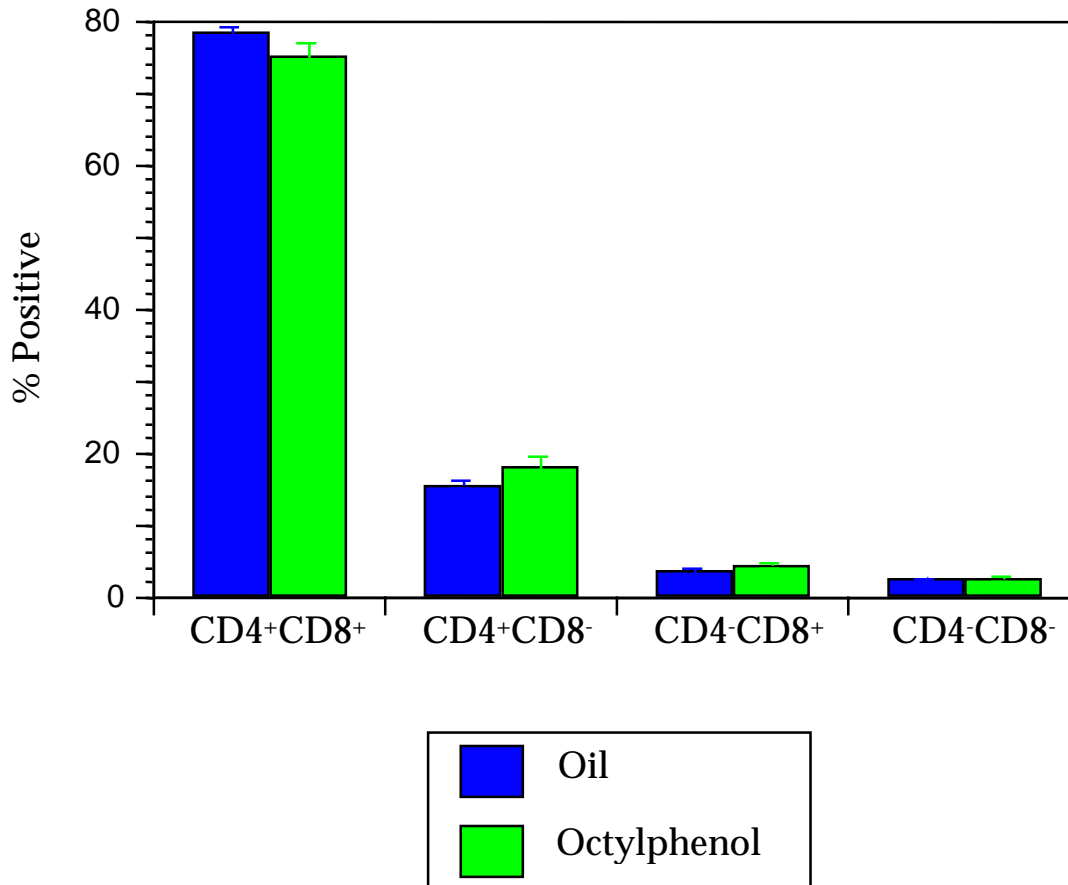
In the models tested, the thymus seemed to be the lymphoid organ that was most sensitive to treatment with octylphenol and genistein. Furthermore, octylphenol treatment resulted in a decrease in the T cells of the thymus and possibly an increase in apoptosis of T cells. The thymus is the site for the development and selection of T cells. Therefore, any alteration in the number of T cells that are able to develop to maturity or in apoptosis, which is necessary for the selection process, could adversely affect the ability of the immune system in fighting infection or could theoretically result in the development of an autoimmune disease. Therefore, future studies should investigate further the effects of octylphenol treatment on the thymus in normal mice and autoimmune mice. Furthermore other models, such as females and males of a different age, and different routes of exposure should be investigated to determine other models in which the thymus may be sensitive to the effects of octylphenol and genistein.

## Effects of Octylphenol Treatment on the Cellularity and Weight of Lymphoid Organs in Orchietomized B/W Mice



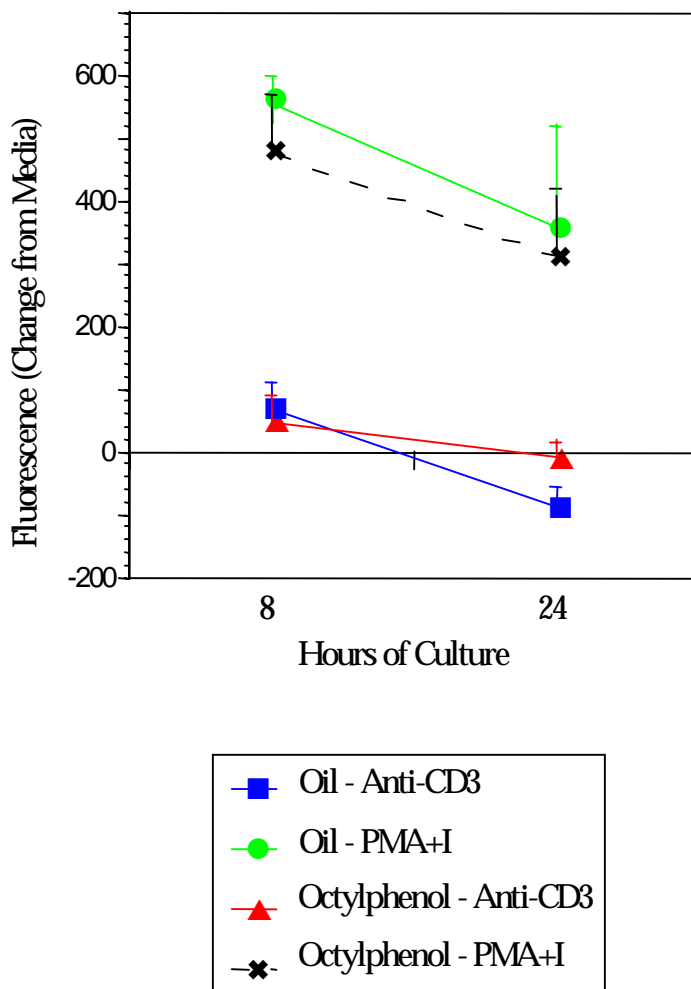
**Fig. 2.1:** Five-month old orchietomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the mice were weighed and the thymus and spleen were removed. a.) Leukocytes were isolated from the spleen and lymphocytes were isolated from the thymus and counted. b.) The spleen and thymus were weighed and the percent body weight was calculated.  $p \leq 0.05$  was considered significant. (n=4 for control, n=5 for octylphenol)

## Effects of Octylphenol Treatment on Thymocyte Subsets of Orchiectomized B/W Mice



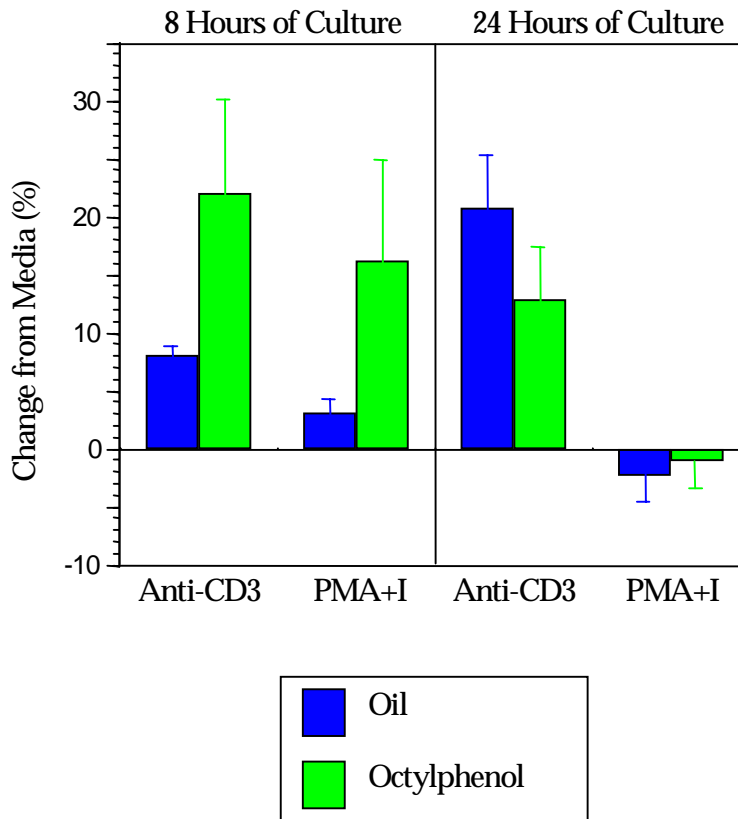
**Fig. 2.2:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the thymocytes were isolated and stained with PE-conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8a antibodies or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.05$  was considered significant. (n=4 for control, n=5 for octylphenol)

### Mitogen-Induced Proliferation of Thymocytes from Octylphenol Treated Orchiectomized B/W Mice



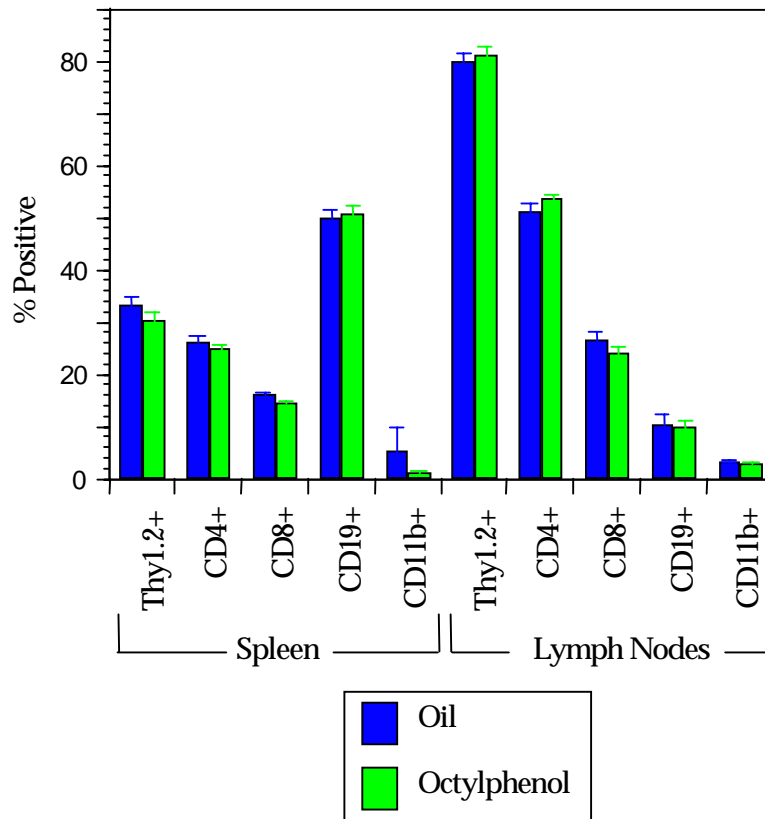
**Fig. 2.3:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the thymocytes were isolated and stimulated with Anti-CD3 antibodies, PMA+I, or media for a baseline. Alamar blue was added to the cells and the fluorescence was read 8 and 24 hours of culture (ex. 530, em. 590, gain=35). The data are presented as the change in fluorescence from the media baseline. Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control, n=5 for octylphenol)

## Activation-Induced Apoptosis of Thymocytes from Octylphenol Treated Orchiectomized B/W Mice



**Fig. 2.4:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the thymocytes were isolated and stimulated with Anti-CD3 antibodies, PMA+I, or media for a baseline. At 8 and 24 hours of culture the thymocytes were stained with Vindelov's propidium iodide solution and fixed with sodium azide. The stained thymocytes were analyzed using an EPIC-XL flow cytometer and the proportion of the cells in the sub G<sub>0</sub> peak was determined and considered apoptotic. The data are presented as the change from the media baseline. Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control, n=5 for octylphenol)

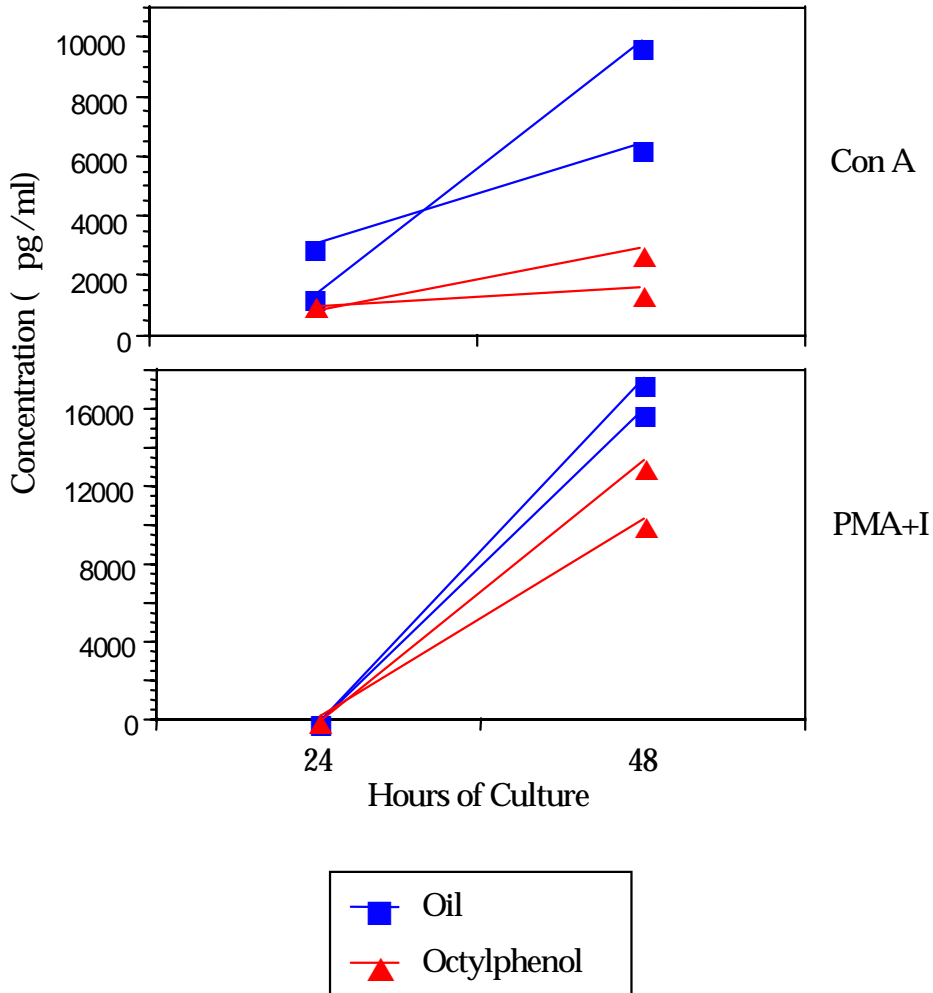
## Effects of Octylphenol Treatment on T cell, B cell, and Macrophage Subsets in Secondary Lymphoid Organs of Orchiectomized B/W Mice



**Fig. 2.5:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and lymph nodes and stained with PE-conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8a antibodies, PE-conjugated anti-CD19 antibodies and FITC-conjugated anti-Thy1.2 antibodies, FITC-conjugated anti-CD11b antibodies, or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.05$  was considered significant. (n=4 for control, n=5 for octylphenol – spleen; n=3 for control, n=5 for octylphenol – lymph nodes)

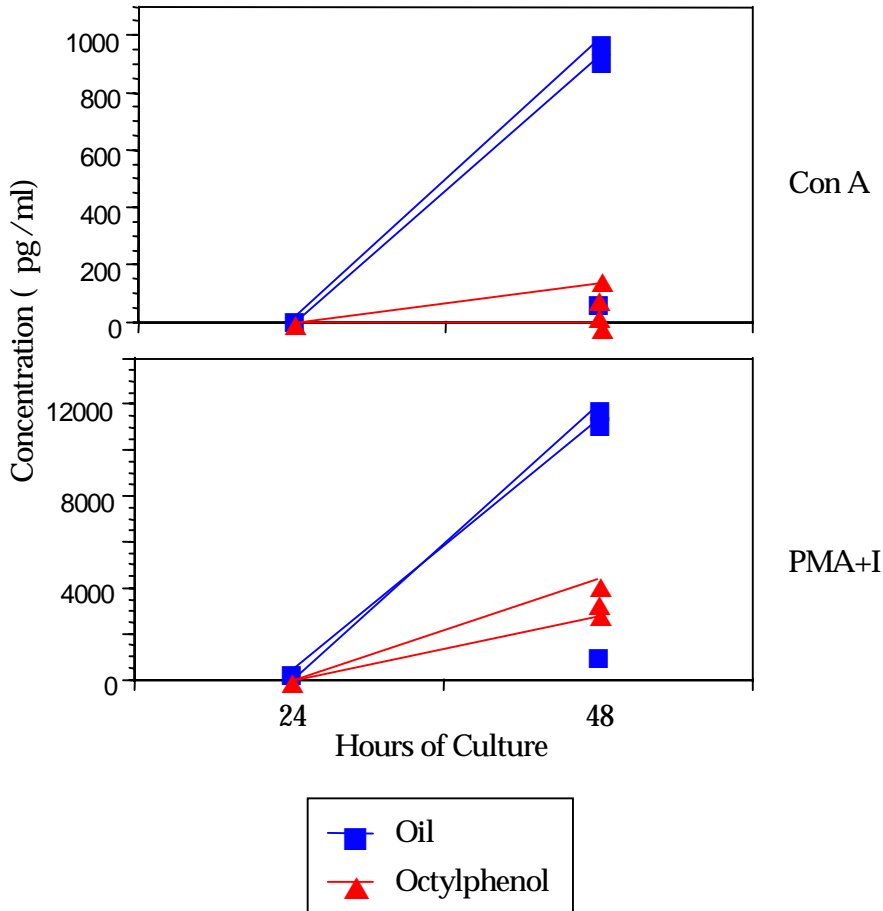


**Effects of Octylphenol Treatment on the Secretion of IFN- $\gamma$   
from Splenic Lymphocytes of Orchiectomized B/W Mice  
Stimulated with Con A and PMA+I**



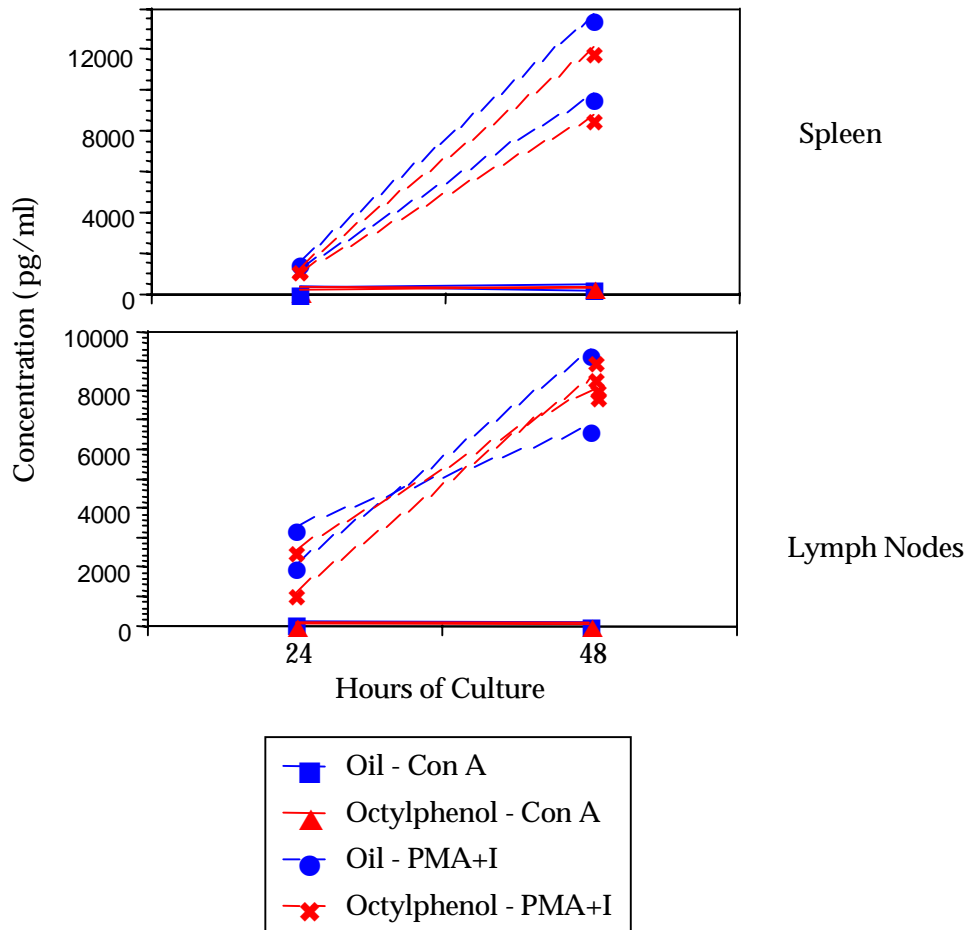
**Fig. 2.6:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and stimulated with Con A or PMA+I. After 24 and 48 hours of culture the supernatants were removed and the concentration of IFN- $\gamma$  within the supernatants was determined by sandwich ELISA.  $p \leq 0.05$  was considered significant. (n=2 for control and octylphenol)

**Effects of Octylphenol Treatment on the Secretion of IFN- $\gamma$  by Lymphocytes from the Lymph Nodes of Orchiectomized B/W Mice Stimulated with Con A and PMA+I**



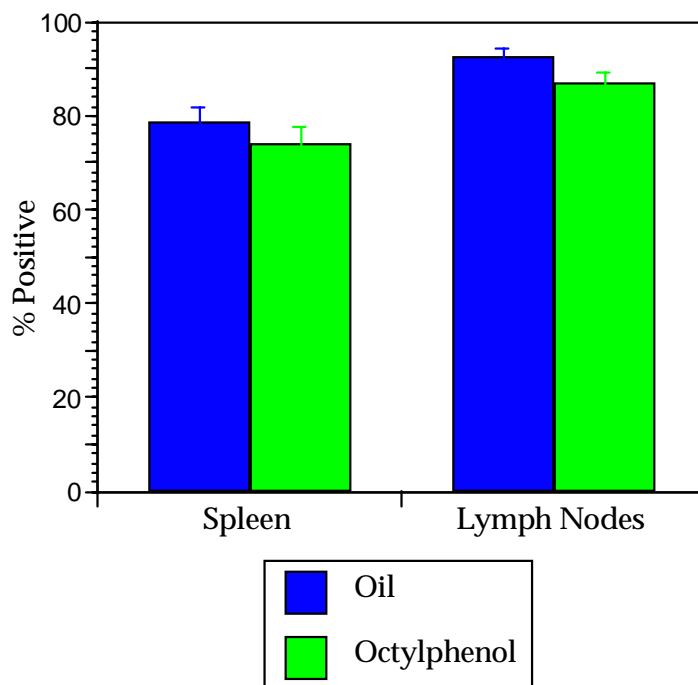
**Fig. 2.7:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the lymph nodes and stimulated with Con A or PMA+I. After 24 and 48 hours of culture the supernatants were removed and the concentration of IFN- $\gamma$  within the supernatants was determined by sandwich ELISA.  $p \leq 0.05$  was considered significant. (n=2 for control and octylphenol at 24 hours; n=3 for control at 48 hours; n=4 for octylphenol at 48 hours)

**Secretion of IL-2 by Stimulated Lymphocytes from the Spleen and Lymph Nodes of Orchiectomized Octylphenol Treated B/W Mice**



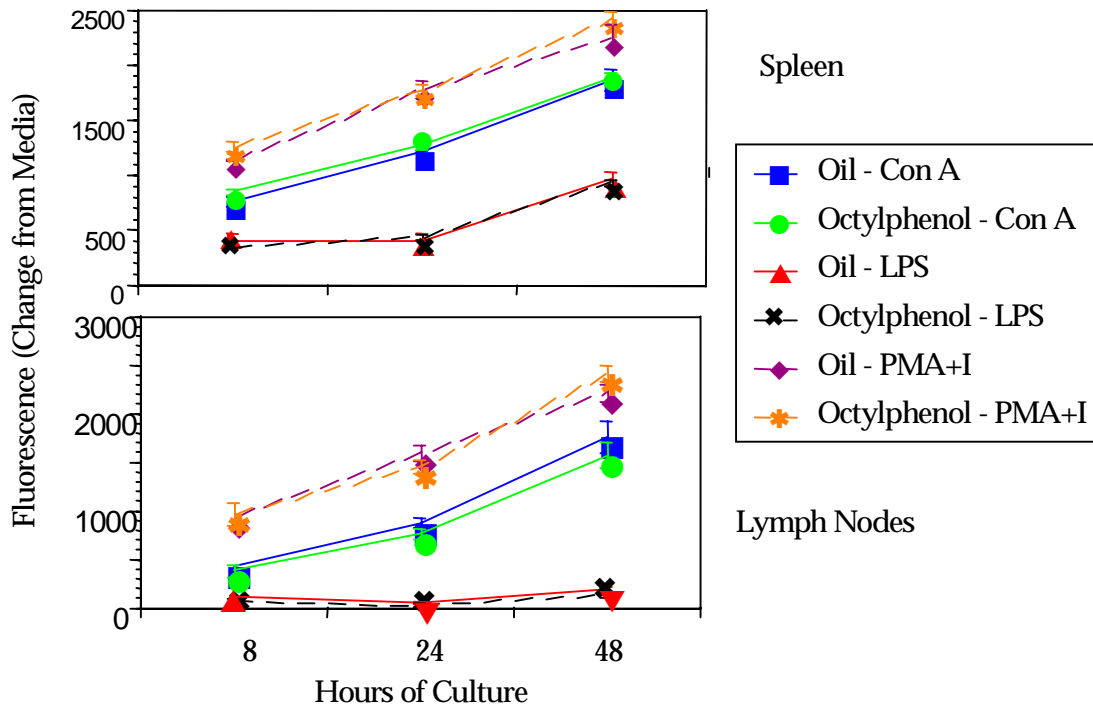
**Fig. 2.8:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and lymph nodes. Lymphocytes from the spleen and lymph nodes were stimulated with Con A or PMA+I. After 24 and 48 hours of culture the supernatants were removed and the concentration of IL-2 within the supernatants was determined by sandwich ELISA.  $p \leq 0.05$  was considered significant. (n=2 for control and octylphenol at 24 and 48 hours - spleen; n=2 for control and octylphenol at 24 hour - lymph nodes; n=4 for control and octylphenol at 48 hours - lymph nodes)

**Effects of Octylphenol Treatment on the Expression of Very Early Activation Antigen, CD69, on Stimulated Cells from Orchiectomized B/W Mice**



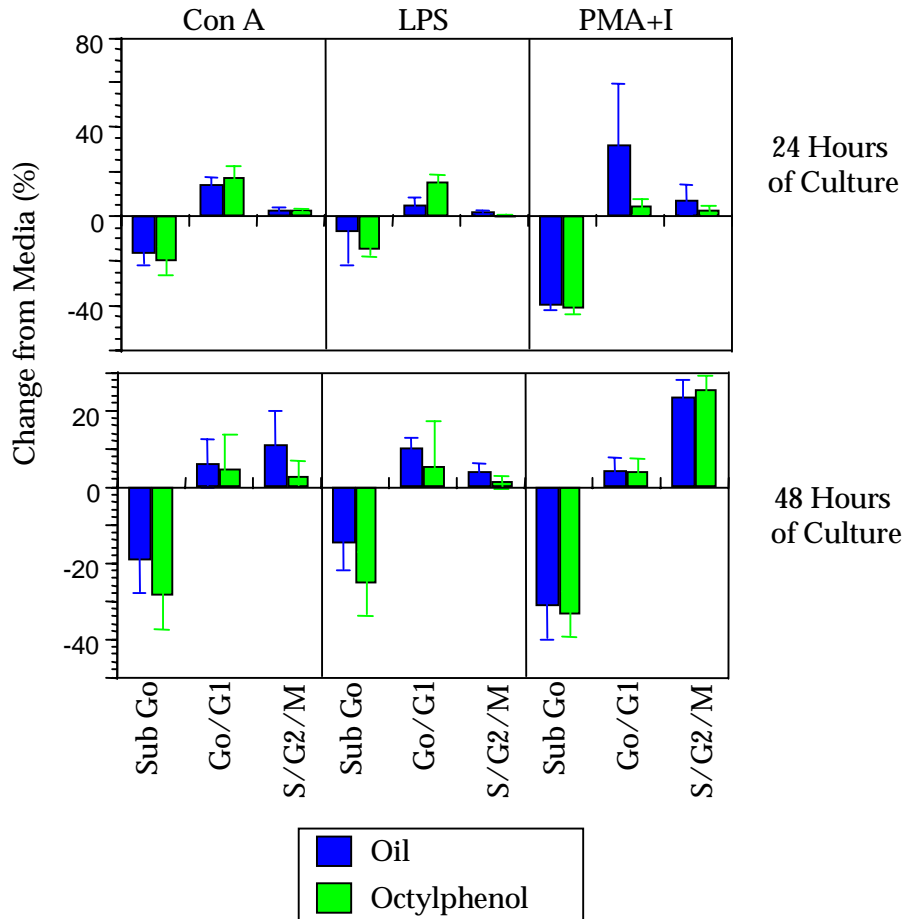
**Fig. 2.9:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and lymph nodes and stimulated with PMA+I. After 6 hours of culture the cells were stained with PE-conjugated anti-CD69 or an isotype matched control. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.05$  was considered significant. (n=4 for control, n=5 for octylphenol – spleen; n=3 for control, n=5 for octylphenol – lymph nodes)

### Mitogen-Induced Proliferation of Splenic and Lymph Node Leukocytes from Octylphenol Treated Orchiectomized B/W Mice



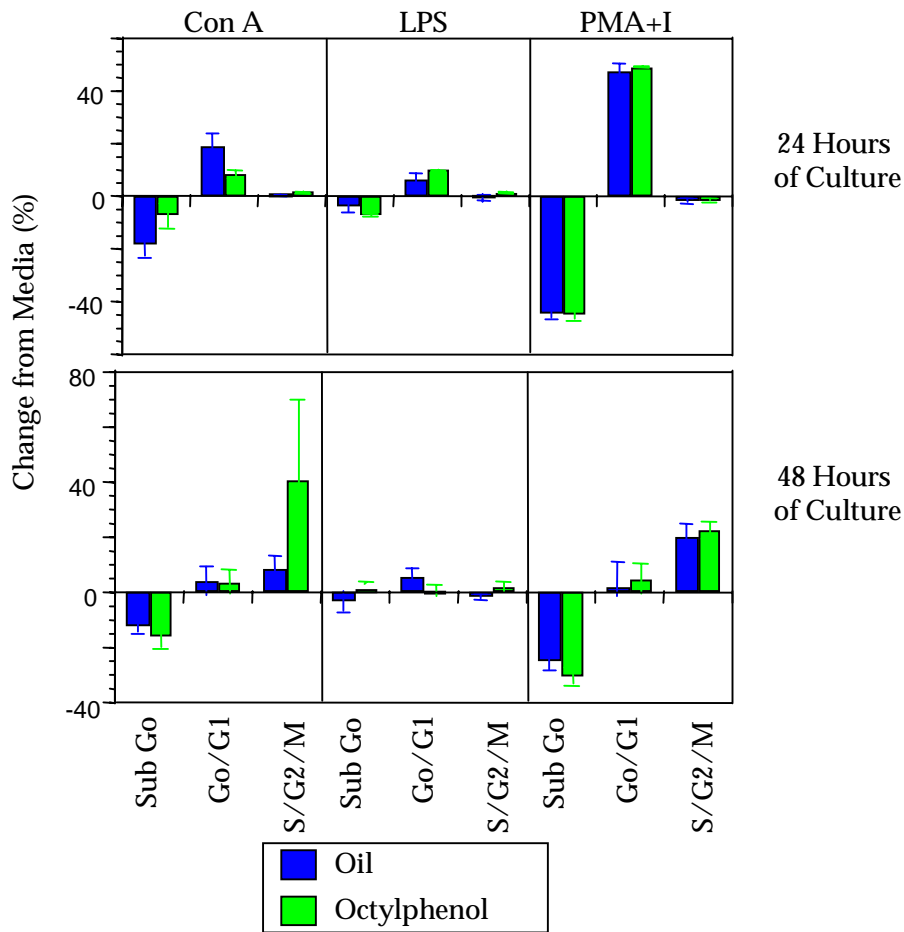
**Fig. 2.10:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and lymph nodes. Leukocytes from the spleen and lymph nodes were stimulated with Con A, LPS, PMA+I, or media for a baseline. Alamar blue dye was added to the cell cultures and the fluorescence was read at 8, 24, and 48 hours of culture (ex. 530, em. 590, gain=35). The data are presented as the change in fluorescence from the media baseline. Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=2 for control and n=3 for octylphenol – spleen at 12 hours; n=4 for control and n=5 for octylphenol – spleen at 24 and 48 hours ; n=1 for control and n=3 for octylphenol – lymph nodes at 12 hours; n=3 for control and n=5 for octylphenol – lymph nodes at 24 and 48 hours)

### Cell Cycle Analysis of Splenic Leukocytes from Octylphenol Treated Orchiectomized B/W Mice at 24 and 48 hours of Culture



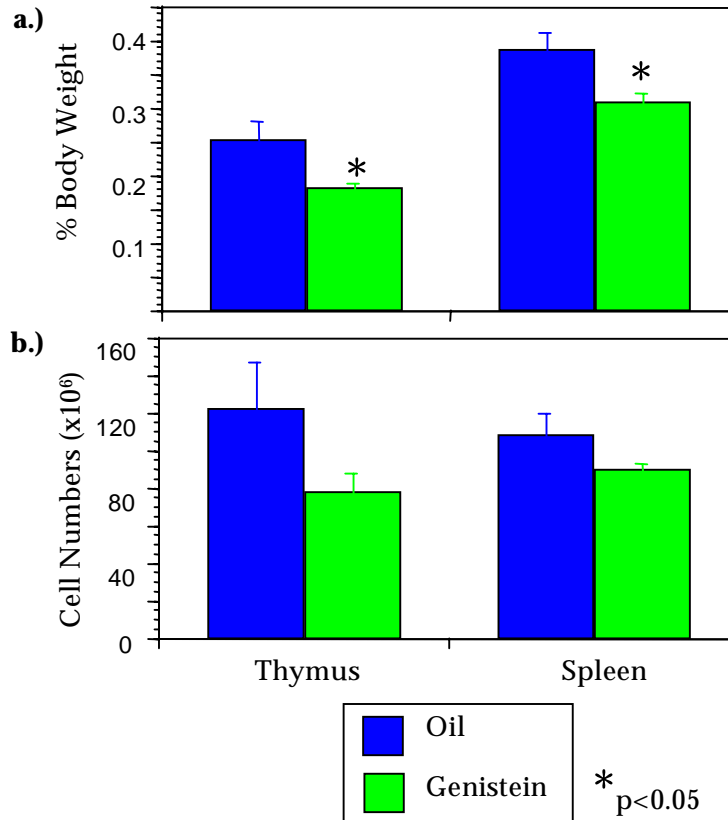
**Fig. 2.11:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and stimulated with Con A, LPS, PMA+I, or media for a baseline. At 24 hours and 48 hours of culture the leukocytes were stained with Vindelov's propidium iodide solution and fixed with sodium azide. The stained leukocytes were analyzed using an EPIC-XL flow cytometer and the proportion of the cells in the sub Go, Go/G1, S, and G2/M peaks was determined. The data are presented as the change from the media baseline in the proportion of cells that are in the sub Go peak (apoptotic cells), Go/G1 peak (resting cells), and S+G2/M peaks (cells entering the cell cycle or actively cycling). Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control and n=5 for octylphenol)

### Cell Cycle Analysis of Leukocytes from the Lymph Nodes of Octylphenol Treated Orchiectomized B/W Mice at 24 and 48 hours of Culture



**Fig. 2.12:** Five-month old orchiectomized B/W males were treated three times a week for three weeks with 10 mg of octylphenol or an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the lymph nodes and stimulated with Con A, LPS, PMA+I, or media for a baseline. At 24 and 48 hours of culture the leukocytes were stained with Vindelov's propidium iodide solution and fixed with sodium azide. The stained leukocytes were analyzed using an EPIC-XL flow cytometer and the proportion of the cells in the sub Go, Go/G1, S, and G2/M peaks was determined. The data are presented as the change from the media baseline in the proportion of cells that are in the sub Go peak (apoptotic cells), Go/G1 peak (resting cells), and S+G2/M peaks (cells entering the cell cycle or actively cycling). Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=2 for control and octylphenol – lymph nodes at 24 hours; n=3 for control and n=5 for octylphenol – lymph nodes at 48 hours)

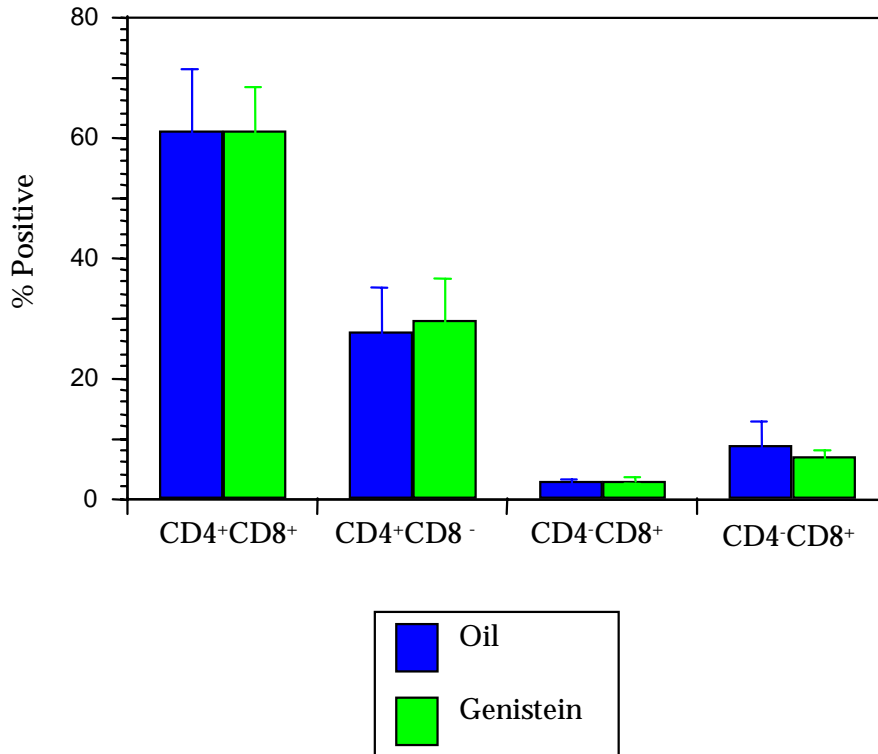
### Effect of Genistein Treatment on the Weight and Cellularity of Lymphoid Organs in C57BL/6 Mice



**Fig. 2.13:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the mice were weighed and the thymus and spleen were removed. a.) The spleen and thymus were weighed and the percent body weight was calculated. b.) The lymphocytes were isolated from the thymus and the leukocytes were isolated from the spleen and counted.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

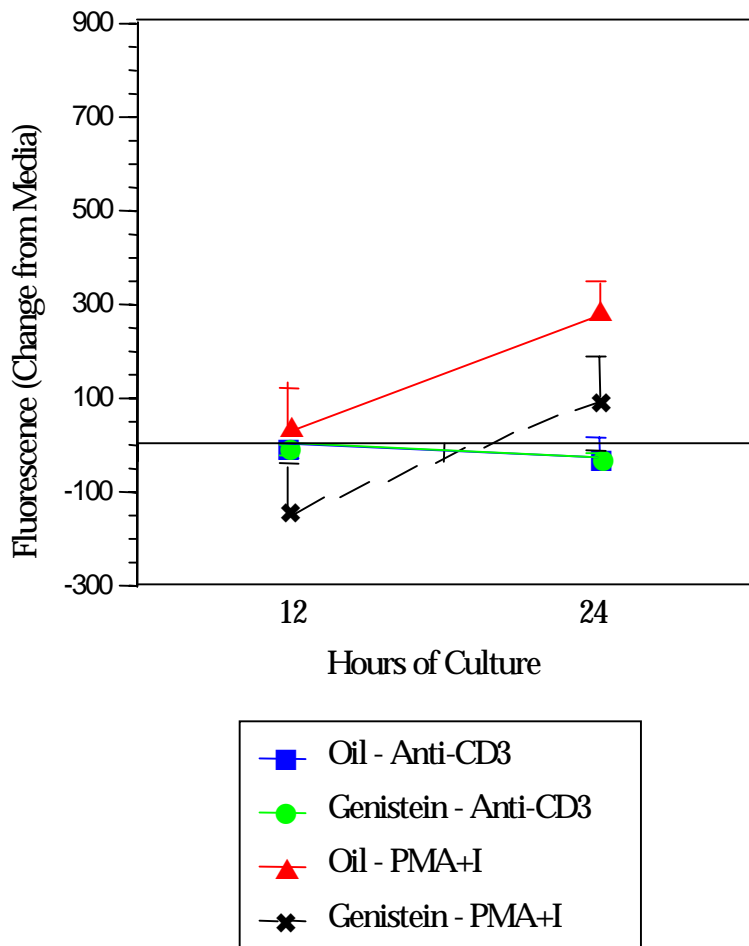


### Effect of Genistein Treatment on the Thymocyte Subsets of C57BL/6 Mice



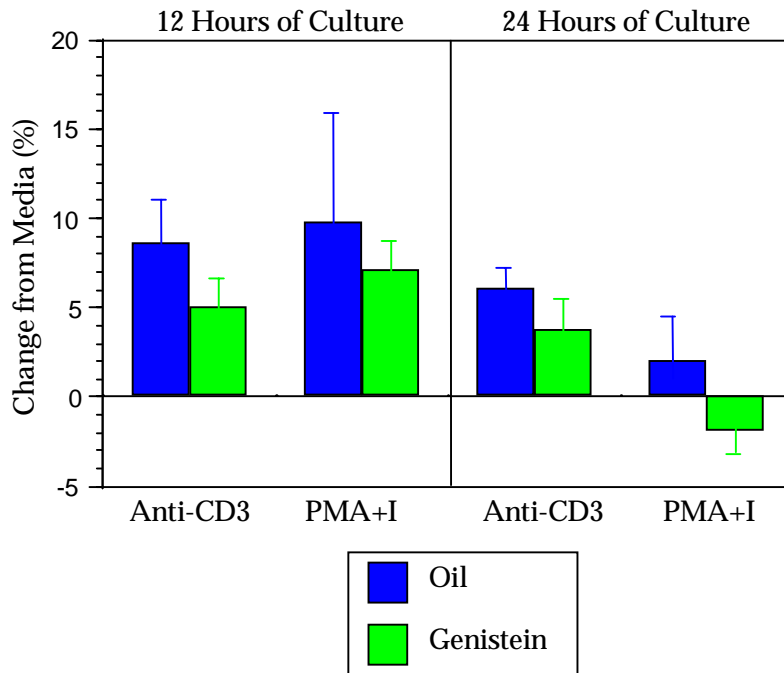
**Fig. 2.14:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the thymocytes were isolated and stained with PE-conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8a antibodies or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

### Mitogen-Induced Proliferation of Thymocytes from Genistein Treated C57BL/6 Mice



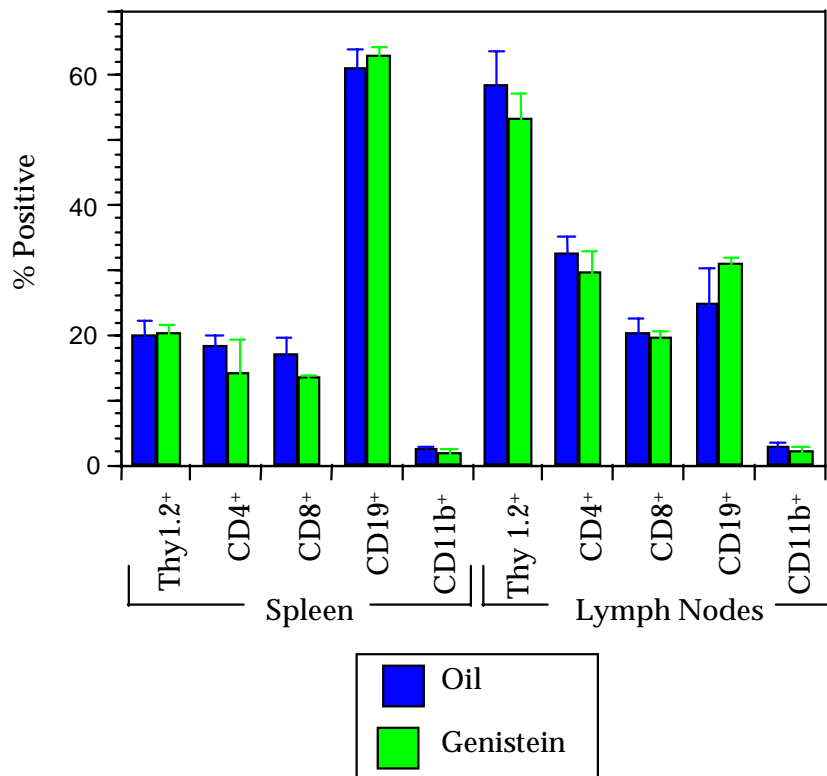
**Fig. 2.15:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the thymocytes were isolated and stimulated with Anti-CD3 antibodies, PMA+I, or media for a baseline. Alamar blue was added to the cells and the fluorescence was read 12 and 24 hours of culture (ex. 530, em. 590, gain=35). The data are presented as the change in fluorescence from the media baseline. Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

### Activation-Induced Apoptosis of Thymocytes from Genistein Treated C57BL/6 Mice



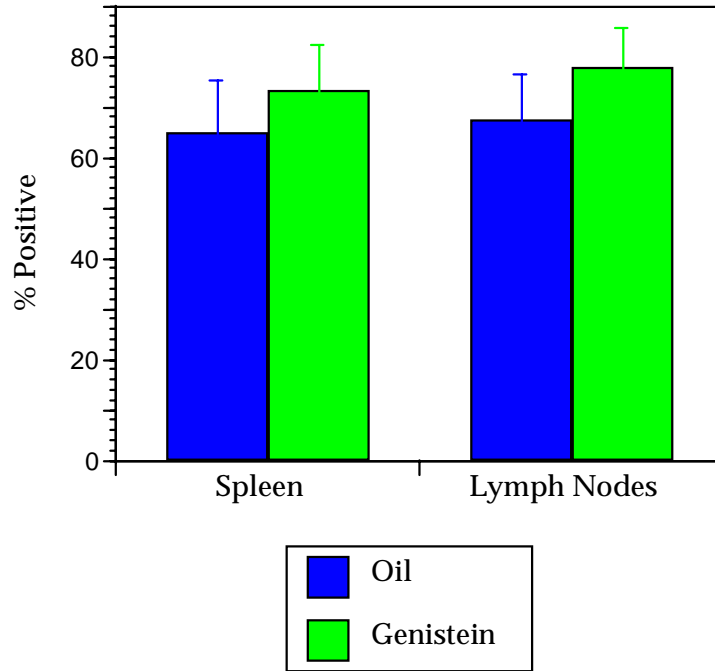
**Fig. 2.16:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the thymocytes were isolated and stimulated with Anti-CD3 antibodies, PMA+I, or media for a baseline. At 12 and 24 hours of culture the thymocytes were stained with Vindelov's propidium iodide solution and fixed with sodium azide. The stained thymocytes were analyzed using an EPIC-XL flow cytometer and the proportion of the cells in the sub Go, Go/G1, S, and G2/M peaks was determined. The data are presented as the change from the media baseline in the proportion of cells that are in the sub Go (apoptotic cells), Go/G1 (resting cells), and S/G2/M (cells entering the cell cycle or actively cycling) peaks. Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

### Effects of Genistein Treatment on Leukocyte subsets in the Spleen and Lymph Nodes of C57BL/6 Mice



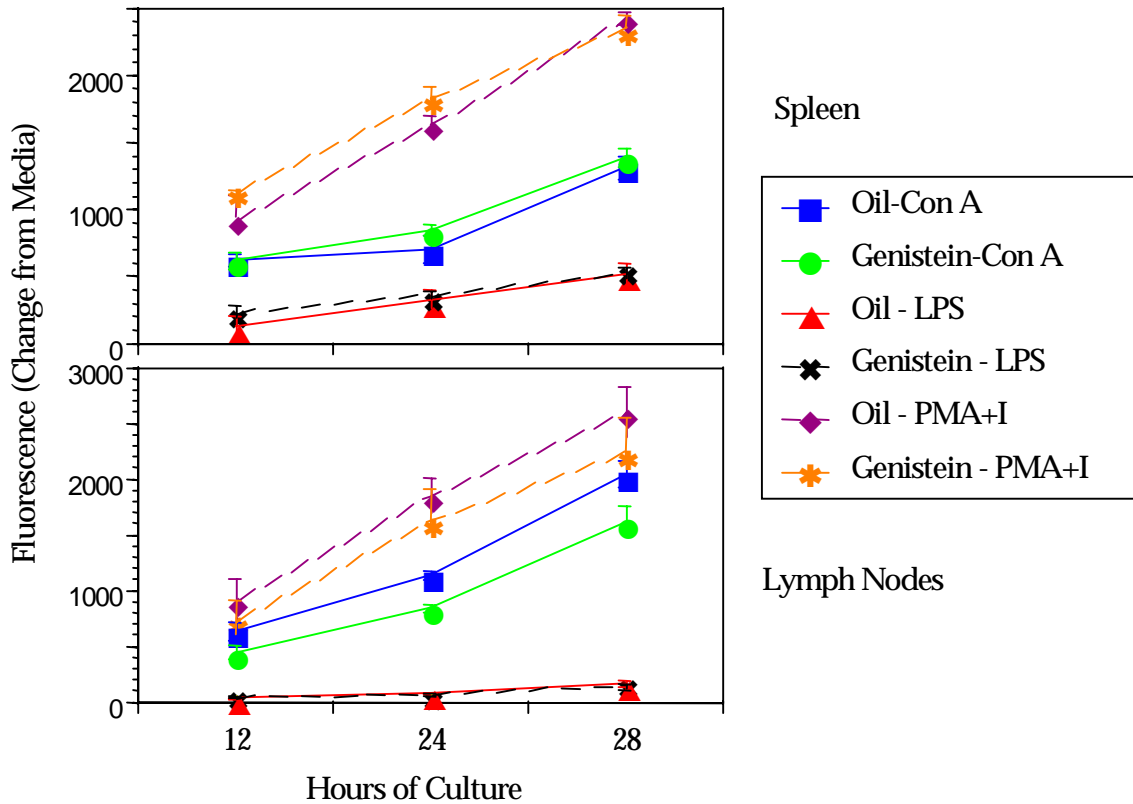
**Fig. 2.17:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the leukocytes were isolated from the spleen and lymph nodes and stained with PE-conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8a antibodies, PE-conjugated anti-CD19 antibodies and FITC-conjugated anti-Thy1.2 antibodies, FITC-conjugated anti-CD11b antibodies, or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

### Effects of Genistein Treatment on the Expression of Very Early Activation Antigen, CD69, in C57BL/6 Mice



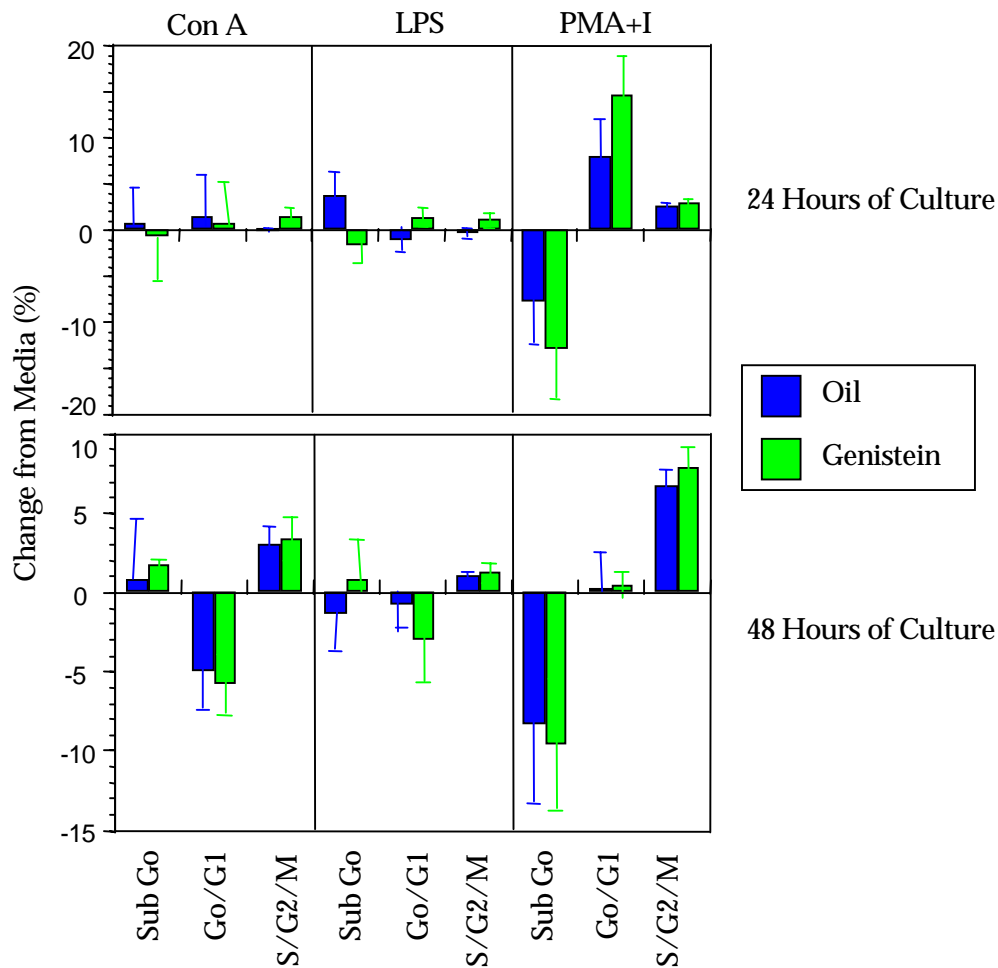
**Fig. 2.18:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the leukocytes were isolated from the spleen and lymph nodes and stimulated with PMA+I. After 6 hours of culture the cells were stained with PE-conjugated anti-CD69 or an isotype matched control. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

## Proliferation of Mitogen-Stimulated Leukocytes from the Spleen and Lymph Nodes of Genistein Treated C57BL/6 Mice



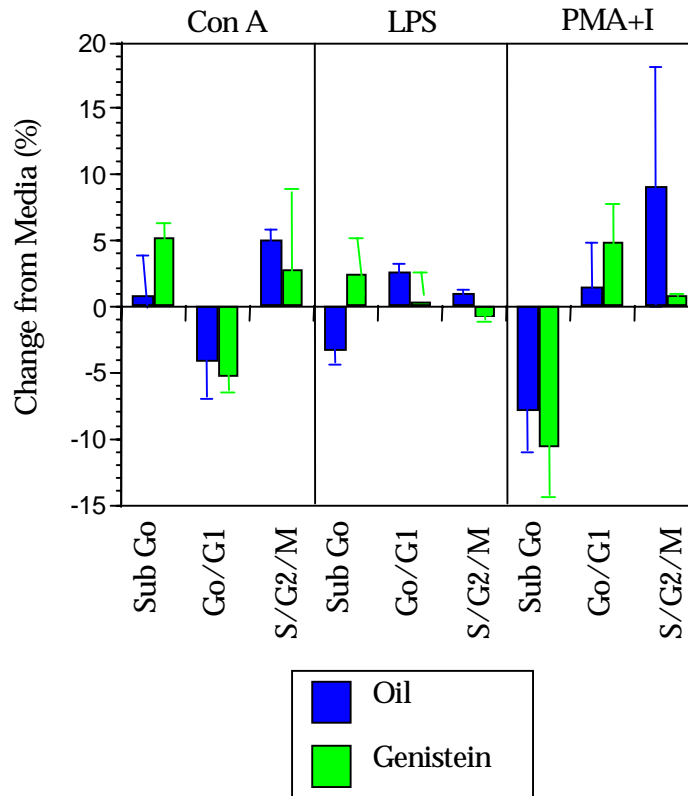
**Fig. 2.19:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the leukocytes were isolated from the spleen and lymph nodes. Leukocytes from the spleen and lymph nodes were stimulated with Con A, LPS, PMA+I, or media for a baseline. Alamar blue dye was added to the cell cultures and the fluorescence was read at 12, 24, and 48 hours of culture (ex. 530, em. 590, gain=35). The data are presented as the change in fluorescence from the media baseline. Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=2 for control and genistein – spleen at 12 hours; n=4 for control and genistein – lymph nodes at 12, 24, and 48 hours, spleen at 24 and 48 hours)

### Cell Cycle Analysis of Splenic Leukocytes from Genistein Treated C57BL/6 Mice at 24 and 48 Hours of Culture



**Fig. 2.20:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the leukocytes were isolated from the spleen and stimulated with Con A, LPS, PMA+I, or media for a baseline. At 24 hours and 48 hours of culture the leukocytes were stained with Vindelov's propidium iodide solution and fixed with sodium azide. The stained leukocytes were analyzed using an EPIC-XL flow cytometer and the proportion of the cells in the sub Go, Go/G1, S, and G2/M peaks was determined. The data are presented as the change from the media baseline in the proportion of cells that are in the sub Go peak (apoptotic cells), Go/G1 peak (resting cells), and S+G2/M peaks (cells entering the cell cycle or actively cycling). Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)

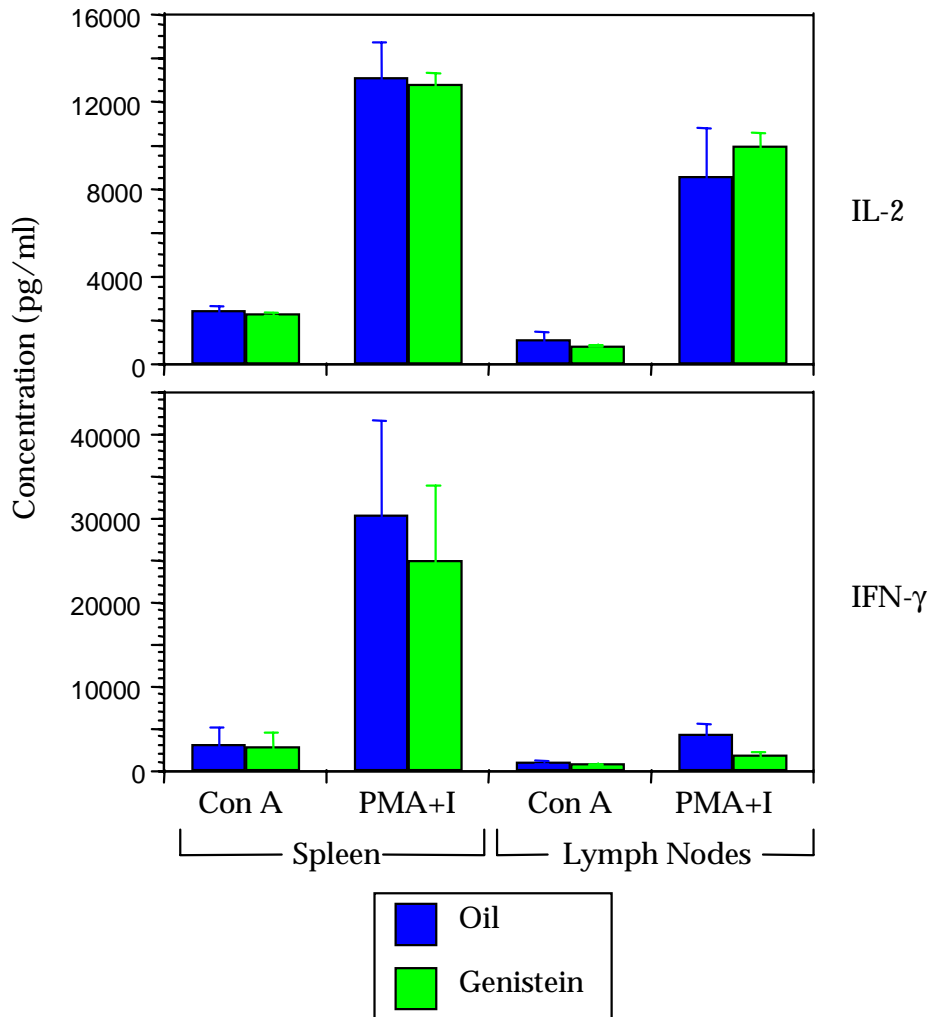
### Cell Cycle Analysis of Leukocytes from the Lymph Nodes at 48 hours of Culture



**Fig. 2.21:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the leukocytes were isolated from the lymph nodes and stimulated with Con A, LPS, PMA+I, or media for a baseline. At 48 hours of culture the leukocytes were stained with Vindelov's propidium iodide solution and fixed with sodium azide. The stained leukocytes were analyzed using an EPIC-XL flow cytometer and the proportion of the cells in the sub Go, Go/G1, S, and G2/M peaks was determined. The data are presented as the change from the media baseline in the proportion of cells that are in the sub Go peak (apoptotic cells), Go/G1 peak (resting cells), and S+G2/M peaks (cells entering the cell cycle or actively cycling). Media baselines are listed in the appendix.  $p \leq 0.05$  was considered significant. (n=4 for control and genistein)



**Effects of Genistein Treatment on the Secretion of IL-2 and IFN- $\gamma$  from Lymphocytes of the Spleen and Lymph Nodes of C57BL/6 Mice**



**Fig. 2.22:** Five-month old C57BL/6 males were fed 0.6 mg of genistein or an ethanol/oil control everyday for three weeks. The day after the last treatment the leukocytes were isolated from the spleen and lymph nodes. Leukocytes from the spleen and lymph nodes were stimulated with Con A or PMA+I. After 48 hours of culture the supernatants were removed and the concentration of IL-2 and IFN- $\gamma$  within the supernatants was determined by sandwich ELISA.  $p \leq 0.05$  was considered significant. (n=4 for control and octylphenol – spleen and lymph nodes with Con A; n=4 for control and n=3 for octylphenol – spleen and lymph nodes with PMA+I)

## **Chapter 3**

### **Dose range studies to determine the effects of short-term oral administration of octylphenol on the thymus of reproductively mature C57BL/6 and B/W males**

#### **a.) Introduction**

Preliminary studies examined the effects of octylphenol on orchietomized male B/W mice and genistein on male C57BL/6 mice in an attempt to determine whether the spleen, thymus, or lymph nodes are morphologically or functionally sensitive to the effects of these endocrine disruptors. The greatest effects were seen in the thymus of the orchietomized B/W mice treated with octylphenol. B/W mice in the octylphenol treatment group exhibited a decrease in thymocyte numbers, without a comparable decrease in thymic to body weight ratios or any change in the proportion of immature or mature thymocytes. Furthermore, a trend of increased apoptosis was seen in anti-CD3 antibody and PMA+I stimulated thymocytes from octylphenol treated mice. Even though no remarkable changes in T cell functions were evident, there was a trend of decreased IFN- $\gamma$  secretion in octylphenol treated mice. Based on the above preliminary data, the connection between a decrease in thymic cellularity and a trend of increased apoptosis of thymocytes caused by octylphenol treatment was explored further in C57BL/6 and B/W males.

The preliminary studies utilized orchietomized males, in order to test a model that we felt would be the most sensitive to the effects of weak estrogenic chemicals. However, orchietomized animals are not the most environmentally relevant models for exposure to octylphenol in humans, wildlife, or the majority of livestock. Both males and females are exposed to octylphenol, and due to differing levels of endogenous estrogens and androgens, the effects on a female could differ from the effects on a male. Furthermore, in females the effects of octylphenol could vary since estrogen levels fluctuate throughout the life cycle of a single female. Also, prenatal or neonatal exposure could have the greatest effects, because the thymus is still developing. In these studies we chose to focus on reproductively mature males because of lower endogenous levels of estrogen in the males and because their gonadal hormones would be fairly stable until the geriatric stage. It is also possible that octylphenol could affect the thymus by means other than estrogenic properties. In this case, the adult male model is as

good as any model for initial examinations. Therefore, further studies utilize intact, reproductively mature male C57BL/6 and B/W mice.

Orchiectomized male B/W mice treated with estrogens have an accelerated onset of autoimmune disease (Roubinian et al., 1978; Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1990, 1993, 1988, 1998; Walker et al., 1994). Signs of autoimmunity, such as dsDNA autoantibodies, which normally do not develop until 10 - 12 months on age, have been noted as early as 3 - 4 months of age in estrogen treated orchiectomized B/W males (Roubinian et al., 1978). The estrogen treated B/W and C57BL/6 mice in our model are euthanized at 3 - 3 ½ months of age. Young B/W males in a pre-autoimmune state were chosen for this study. These mice are most likely too young to have developed signs of autoimmune disease; however, the effects of octylphenol on the B/W model are not known. Therefore, autoimmune parameters will be analyzed.

The dose of octylphenol in the preliminary studies was based on doses that have been shown to cause estrogenic changes on the reproductive system of male rats, when injected subcutaneously (Blake and Boockfor, 1997). Although, oral administration may decrease the effect of octylphenol compared to subcutaneous injection, the 10 mg dose of octylphenol is already much higher than would normally be encountered in the environment; therefore, the dose was not increased. Furthermore, as stated before, the environmental exposure is much lower than the 10 mg dose. To extrapolate from a murine dose to a human dose, a 10 mg dose for a 25 g mouse is equal to a 24-28 g dose for a 60-70 kg human. Therefore, a range of doses (0.1 mg, 1 mg, and 10 mg) was utilized in an attempt to characterize effects seen at a high dose and determine whether more environmentally relevant doses are still able to cause changes.

## **b.) Specific Aims**

I) The primary aim of this study was to determine whether oral dosing of octylphenol at 0.1 mg, 1 mg, or 10 mg alters the morphology or function of the thymus in intact, adult B/W and C57BL/6 males. The following were examined:

### a.) Morphology of Primary Lymphoid Organs:

- ❖ Thymus weight and cellularity
- ❖ Proportion of immature and mature thymocytes
- ❖ Bone marrow cellularity to determine whether a decrease in thymic cellularity is due to a decrease in thymocyte precursor cells from the bone marrow

b.) Function of Thymus:

- ❖ Proportion of immature and mature thymocytes to determine whether proper development of T cells is occurring
- ❖ Apoptosis of unstimulated and stimulated thymocytes to determine whether there is proper signaling for apoptosis

c.) Autoimmune Parameters:

- ❖ Autoantibody production
- ❖ Histopathologic examination of the kidneys, liver and salivary glands to determine whether proper selection is occurring to prevent autoimmune changes

d.) Morphology of Secondary lymphoid organs:

- ❖ Splenic weight and leukocyte cellularity
- ❖ T cell/B cell proportions to determine any alteration in the migration of mature T cells out of the thymus into the spleen

II) The secondary aim of this study is to determine whether octylphenol acts in an estrogenic manner in C57BL/6 or B/W intact, adult males

a.) 17- $\beta$ -estradiol will be utilized as a positive control for all studies

b.) Testis and seminal vesicles will be weighed to determine reproductive changes

**c.) Materials and Methods**

**Mice:** Eight to ten-week old C57BL/6 males were purchased from Charles River (Wilmington, MA) and eight to ten-week old B/W males were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed, fed, and watered as in Chapter 2.

**Octylphenol and 17 $\beta$ -estradiol treatment:** One week after arrival the B/W and C57BL/6 males were fed 10 mg, 1 mg, or 0.1 mg of octylphenol, 6  $\mu$ g or 9  $\mu$ g of 17 $\beta$ -estradiol, or an ethanol/oil control three times a week for three weeks. Octylphenol (Sigma, St. Louis, MO) was dissolved in ethanol to make a stock concentration of 10 mg/20  $\mu$ l. The dose of 17 $\beta$ -estradiol was based on a dose that has been shown to induce thymic atrophy in short-term studies by subcutaneous injection (Ansar Ahmed et al., 1985b). The dose was increased in an attempt to account for a decrease in activity due to oral administration. A 1:10 dilution in ethanol of the stock solution resulted in a 1 mg/20  $\mu$ l solution, and a 1:10 dilution

of the 1 mg/20  $\mu$ l solution resulted in a 0.1 mg/20  $\mu$ l solution. Twenty microliters of each solution were mixed with 10  $\mu$ l of autoclaved corn oil (ICN, Aurora, OH) and fed to the mice with a disposable tip on a micropipetter three times a week for three weeks. Two concentrations of 17 $\beta$ -estradiol (Sigma) were made: 6  $\mu$ g/20  $\mu$ l and 9  $\mu$ g/20  $\mu$ l. Twenty microliters of the 6  $\mu$ g/20  $\mu$ l solution were mixed with 10  $\mu$ l of corn oil and fed to C57BL/6 males as a positive control and twenty microliters of the 9  $\mu$ g/20  $\mu$ l solution were mixed with 10  $\mu$ l of corn oil and fed to B/W males as a positive control. A high dose of 17 $\beta$ -estradiol was fed to the B/W mice because they weighed approximately one third more than the C57BL/6 mice at the start of dosing. Negative control mice were fed 20  $\mu$ l of ethanol mixed with 10  $\mu$ l of corn oil. All solutions were made fresh each day of feeding. Three days after the last treatment the mice were killed by cervical dislocation.

**Serum collection and detection of dsDNA antibodies:** Mice were bled retro-orbitally prior to euthanization. Serum was collected and kept frozen until tested for dsDNA antibodies. The dsDNA antibodies were detected in the serum by standard ELISA techniques as previously reported (Verthelyi and Ansar Ahmed, 1994, 1997, 1998). Briefly, 96-well flat bottom plates (High-binding EIA/RIA plate, Costar, Cambridge, MA) were coated with 2mg/ml methylated bovine serum albumin (mBSA, Sigma) in PBS. Plates were then incubated with calf thymus dsDNA overnight at 4°C. Residual ssDNA was removed by incubation with S1 nuclease (Promega, Madison, WI) and then the wells were blocked with PBS containing 2% bovine serum albumin (BSA, Fisher Scientific, Pittsburg, PA). Mouse sera was diluted in PBS containing 10% heat-inactivated adult bovine serum (Sigma) and added to the wells. After incubation and washing, alkaline phosphatase conjugated goat anti-mouse immunoglobulin (heavy chain specific, Caltag, South San Francisco, CA) diluted in 10% adult bovine serum PBS was added to each well. Plates were washed and developed with p-nitrophenylphosphate in diethanolamine buffer (Sigma). Plates were read at 405 nm on a Molecular Devices kinetic plate reader (Molecular Devices, Menlo Park, CA). Positive anti-dsDNA controls contained sera from MRL/lpr or B/W mice and negative controls contained either known negative sera or all the chemicals but the serum. The absorbance for the negative controls was subtracted from the test samples. The absorbance of the test sera was analyzed as the percent of the positive controls per plate [(O.D.-negative control/positive control)\*100].

**Lymphoid tissue collection and processing:** The mice were weighed and killed by cervical dislocation. The percent body weight of the spleen and thymus

were determined as described above in Chapter 2. The spleen and thymus were then processed and the cellularity determined as described above in Chapter 2. The bone marrow was washed from the left femur by injecting complete media into the cavity with a syringe and 23-gauge needle. The erythrocytes were removed from the bone marrow cell suspensions by lysis in ACK lysis buffer as previously described for the spleen cells and were then washed in complete RPMI media. The bone marrow cells were then counted and the viability was assessed by trypan blue exclusion. All cells were adjusted to a density of  $5 \times 10^6$  cells/ml in complete RPMI and kept at 4°C until utilized in assays.

**Non-lymphoid tissue collection and processing:** The testis, seminal vesicles, kidneys, liver, and salivary glands were removed from each mouse for histopathological examination. The testis and seminal vesicles were weighed and the percent body weight was noted. All tissues were fixed in 10% buffered formalin (Fisher) and examined in a blinded study by a board-certified pathologist.

**Flow cytometric analysis of cell markers:** Cells from the thymus and spleen were stained with appropriate monoclonal antibodies and analyzed by flow cytometry as described in Chapter 2. The following antibodies were utilized in these studies: fluorescein isothiocyanate (FITC)-conjugated anti-CD8a (53-6.7), anti-Thy1.2 (CD90, G7), and rat IgG<sub>2a</sub> isotype control (R35-95); phycoerythrin (PE)- conjugated anti-CD4 (RM4-5), anti-CD19 (1D3), and rat IgG<sub>2a</sub> isotype control (R35-95); and coy-chrome-conjugated anti-AB-TCR (H57-597) and hamster IgG isotype control (G235-2356). All monoclonal antibodies were purchased from Pharmingen (San Diego, CA).

**Detection of apoptosis:** The percent of freshly isolated and stimulated thymocytes undergoing apoptosis was determined by 7-aminoactinomycin-D (7-AAD) fluorescence as previously described (Schemed et al., 1994). Recent studies in our laboratory have shown that the 7-AAD technique is reliable and sensitive in identifying apoptotic thymocytes, and in particular thymocytes in the early stages of apoptosis (Donner et al., 1999). Briefly, for apoptosis of freshly isolated thymocytes, 100  $\mu$ l of 7-AAD (Molecular Probes, Eugene, OR) at 20  $\mu$ g/ml in incomplete media was added to  $5 \times 10^5$  thymocytes suspended in 100  $\mu$ l of incomplete media. The cells were incubated with 7-AAD in the dark for 20 minutes at 4°C and then analyzed by flow cytometry. Negative controls are incubated in incomplete media without 7-AAD. Thymocytes were also cultured for 4, 12, and 24 hours and the percent of apoptotic thymocytes at each time point was detected as above. Briefly, one hundred microliters of thymocytes at a

density of  $5 \times 10^6$  cells/ml were added to 96-well round bottom plates. Thymocytes were stimulated with 100  $\mu$ l of complete RPMI media, complete RPMI media containing 15  $\mu$ g/ml of anti-CD3 antibodies (YCD3, purified in our lab), or complete RPMI media containing 5 ng/ml of phorbol myristate acetate (Sigma) and 0.25  $\mu$ g/ml of ionomycin (Calbiochem, La Jolla, CA) (PMA+I). After 4, 12, and 24 hours of incubation, the cells were pelleted at 300 X g for 6 minutes and resuspended in 100  $\mu$ l of incomplete media. Thymocytes were stained with 7-AAD and analyzed on the flow cytometer as described above. The percent of 7-AAD uptake is directly related to the extent of apoptosis. Therefore, the 7-AAD<sup>dull</sup> cells are considered live, the 7-AAD<sup>intermediate</sup> cells are considered early apoptotic, and the 7-AAD<sup>bright</sup> cells are considered late apoptotic/necrotic. The 7-AAD<sup>intermediate</sup> and 7-AAD<sup>bright</sup> are combined to determine the proportion of the total dying population (7-AAD<sup>intermediate+bright</sup>). The data are represented as the change from the media baseline of the anti-CD3 and PMA+I stimulated thymocytes in each of the 7-AAD<sup>dull</sup>, 7-AAD<sup>intermediate</sup>, 7-AAD<sup>bright</sup>, and 7-AAD<sup>intermediate+bright</sup> proportions of the population.

**Statistics:** The MIXED procedure of SAS (SAS ver. 6.12, SAS Institute, Cary, NC) was used to perform a mixed effects, repeated measures ANOVA on the 7-AAD apoptosis data. A first order autoregressive model was used to model covariance among repeated measurements from a mouse. Single degree of freedom contrasts were utilized to determine whether any of the doses of octylphenol or 17 $\beta$ -estradiol differed from control. The apoptosis data were analyzed as the change from the media baseline. All other data were analyzed utilizing the General Linear Model procedure of SAS to perform a one-way ANOVA. Single degree of freedom contrasts were utilized to determine whether any of the doses of octylphenol or 17 $\beta$ -estradiol differed from control. Because of the multiple comparisons, Bonferonni correction was used to hold the experimentwise error rate to 0.05 (comparisonwise alpha = 0.01, therefore p<0.01 was considered significant).

#### **d.) Results**

**Effect of octylphenol on the morphology of the thymus:** The morphology of the thymus was not altered by treatment with 0.1 mg, 1 mg, or 10 mg of octylphenol in either the C57BL/6 or the B/W males. The percent body weight of the thymus (Fig 3.1), the thymocyte cellularity of the thymus (Fig 3.2), and the proportion of immature (CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup>) thymocytes and presumably mature (CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup>) thymocytes (Fig 3.3) was not significantly altered by treatment with any dose of octylphenol. The 17 $\beta$ -estradiol treatment resulted in a significant decrease in thymocyte cellularity in

B/W males, but did not alter the thymocyte cellularity of C57BL/6 males (Fig 3.2). The percent body weight of the thymus and the proportion of immature and mature thymocytes were not effected by 17 $\beta$ -estradiol treatment in either strain (Fig 3.1, 3.3).

**Effect of octylphenol on apoptosis of thymocytes:** The level of apoptosis in freshly isolated thymocytes and the kinetics of apoptosis of cultured thymocytes stimulated with anti-CD3 antibodies or PMA+I were studied. Neither treatment with 0.1, 1, or 10 mg of octylphenol, nor 17 $\beta$ -estradiol treatment affected the amount of apoptosis in freshly isolated thymocytes in C57BL/6 or B/W males as assessed by 7-AAD fluorescence (Fig 3.4). The kinetic data were analyzed and reported as the changes in the proportions from media of the anti-CD3 and PMA+I stimulated thymocytes in the live (7-AAD<sup>dull</sup>), early apoptotic (7-AAD<sup>intermediate</sup>), late apoptotic/necrotic (7-AAD<sup>bright</sup>), and total dying (7-AAD<sup>intermediate+bright</sup>) cells populations. Anti-CD3 stimulated apoptosis of thymocytes was not affected by *in vivo* treatment with any dose of octylphenol or 17 $\beta$ -estradiol in the B/W males (Fig 3.5, 3.6). However, at 24 hours of culture, the thymocytes from the C57BL/6 males treated with 0.1 mg, 1 mg, and 10 mg of octylphenol and 17 $\beta$ -estradiol exhibit a significantly greater decrease from media in the proportion of live (7-AAD<sup>dull</sup>) cells (Fig 3.7) and a significantly greater increase from media in the proportion of total dying (7-AAD<sup>intermediate+bright</sup>) cells upon anti-CD3 stimulation than the controls (Fig 3.8). Furthermore, the 17 $\beta$ -estradiol treatment also resulted in a significantly greater increase from media at 24 hours in the proportion of late apoptotic/necrotic (7-AAD<sup>bright</sup>) cells upon anti-CD3 stimulation (Fig 3.8). At 12 hours, *in vivo* treatment with 0.1 mg of octylphenol resulted in a significantly greater increase from media in the proportion of late apoptotic/necrotic (7-AAD<sup>bright</sup>) cells upon anti-CD3 stimulation (Fig 3.8). Altered apoptotic responses were also seen in thymocytes from treatment groups of both the C57BL/6 and B/W males in response to PMA+I stimulation (Fig 3.9-3.12). At 24 hours, thymocytes from B/W mice treated *in vivo* with 0.1 mg of octylphenol or 17 $\beta$ -estradiol and from C57BL/6 males treated *in vivo* with 0.1 mg octylphenol, 1 mg of octylphenol, or 17 $\beta$ -estradiol exhibited a significantly greater increase in the proportion of early apoptotic (7-AAD<sup>intermediate</sup>) cells than thymocytes from control mice (Fig 3.10, 3.12).

**Effect of octylphenol on bone marrow cellularity:** The cellularity of the bone marrow was not affected by treatment with any dose of octylphenol in either the C57BL/6 or B/W males (Fig 3.13). The 17 $\beta$ -estradiol treatment did



cause a significant decrease in the bone marrow cellularity in B/W males, but not in C57BL/6 males (Fig 3.13).

**Effect of octylphenol on dsDNA autoantibody production:** The production of anti-dsDNA autoantibodies was not affected by treatment with 0.1 mg, 1 mg, or 10 mg of octylphenol or 17 $\beta$ -estradiol in the C57BL/6 or B/W mice (Fig 3.14). Both the C57BL/6 and B/W mice exhibited detectable levels of IgM isotype anti-dsDNA antibodies. The average absorbance for the B/W males was higher than the average absorbance for the C57BL/6 males. The levels detected in the treatment groups did not differ from the controls in either strain (Fig 3.14). The level of IgG isotype anti-dsDNA antibodies, also, did not differ between the control and the treatment groups in either strain (Fig 3.14).

**Effect of octylphenol on non-lymphoid tissue:** Treatment with 0.1 mg, 1 mg, or 10 mg of octylphenol or 17 $\beta$ -estradiol did not cause pathological changes in the liver, kidneys, or salivary glands of the C57BL/6 (n=30) or B/W males (n=20). The kidneys and salivary glands were found to be normal in all treatment groups from both strains. In one out of six C57BL/6 mice in the control and 1 mg octylphenol treatment groups, in 1 out of 5 in the 0.1 mg octylphenol treatment group, and in 2 out of five mice in the 17 $\beta$ -estradiol treatment group, small foci of neutrophils, macrophages, or lymphocytes were found in the liver. However, they were diagnosed as normal. The liver was found to be normal in all the treated and control B/W mice examined, suggesting that these compounds were not hepatotoxic.

**Effect of octylphenol on the morphology of the spleen:** In both the C57BL/6 and B/W males, the morphology of the spleen was not altered by treatment with 0.1 mg, 1 mg, or 10 mg of octylphenol. The percent body weight of the spleen (Fig 3.15) and the number of splenic leukocytes (Fig 3.16) were not altered by treatment with any dose of octylphenol. The proportion of Thy1.2<sup>+</sup> or  $\alpha\beta$ -TCR<sup>+</sup> T cells and CD19<sup>+</sup> B cells was also unaffected by treatment with octylphenol (Fig 3.17). In contrast the percent body weight of the spleen was significantly increased by treatment with 17 $\beta$ -estradiol in the C57BL/6 males, but not in the B/W males (Fig 3.15). However, neither an increase in the number of splenic lymphocytes, nor any change in the proportion of Thy1.2<sup>+</sup> or  $\alpha\beta$ -TCR<sup>+</sup> T cell or CD19<sup>+</sup> B cells was seen with 17 $\beta$ -estradiol treatment in either strain (Fig 3.16, 3.17).

**Effect of octylphenol on reproductive tissue:** The percent body weight of the testis and seminal vesicles were measured to determine any estrogenic effects

on the reproductive tissue. Neither the testis nor seminal vesicle weights were affected by the 0.1 mg, 1 mg, or 10 mg dose of octylphenol in either the C57BL/6 or B/W males (Fig 3.18, 3.19). Treatment with 17 $\beta$ -estradiol in the C57BL/6 males significantly decreased the % body weight of the seminal vesicles, but not of the testis (Fig 3.18). Neither the seminal vesicles weights nor the testis weights were affected by 17 $\beta$ -estradiol in the B/W males (3.19). The testis and seminal vesicles from all doses of octylphenol and 17 $\beta$ -estradiol were found normal by histopathological examination.

#### **e.) Discussion**

The feeding of 0.1 mg, 1 mg, or 10 mg of octylphenol did not alter the morphology or function of the thymus, as we assessed it, in either the B/W or C57BL/6 males. The decrease in cellularity, which was seen with 10 mg octylphenol treatment of B/W males in preliminary studies, was not seen in these studies in either strain. However, there are distinct differences in the models utilized in the two studies. The preliminary studies utilized orchietomized B/W males in order to increase the sensitivity to detecting the weak estrogenic effects of octylphenol. The present study utilized intact males because it is a more relevant model for the majority of animals exposed to octylphenol. Previous studies in B/W mice have indicated that testosterone has counter-active effects on the estrogenic acceleration of autoimmune disease. In B/W mice it has been shown that castration in males results in acceleration of the disease, whereas testosterone treatment in females results in amelioration of the disease (Melez et al, 1978). Also, the endocrine system is closely regulated by an intricate feedback system, which includes serum and site specific levels of the hormones, and estrogens are produced by the conversion of androgens to estrogens. Therefore, any changes in the androgen levels may affect the conversion of androgens to estrogens through the feedback system. Furthermore, the age of the animals differed between studies. The B/W mice treated in the preliminary studies were 5 months of age and the B/W and C57BL/6 mice utilized in the present study were 2 to 2 ½ months of age. The differences in the models utilized may account for the differing results seen from the same treatment.

The 0.1 mg, 1 mg, and 10 mg doses of octylphenol did not result in any significant changes in the parameters tested, other than apoptosis of thymocytes. Kinetic studies of stimulated thymocyte apoptosis did note significant changes in certain apoptotic or live populations at particular time points. However, no overall change in the kinetics of apoptosis was noted. No changes were noted in the spleen or bone marrow as a result of octylphenol treatment, nor were any

signs of accelerated onset of autoimmune disease noted in the histopathology of the kidneys, liver, or salivary glands or in the level of IgM or IgG anti-dsDNA antibodies in the serum. IgM anti-dsDNA autoantibodies were detected in the serum of both strains, although higher in the B/W than the C57BL/6 mice, but short-term treatment with neither estrogen nor octylphenol were able to accelerate the production of the autoantibodies or the isotype switch to IgG. However, the mice were only 3 – 3 ½ months old by the end of the treatment, which is the earliest that autoimmune parameters, such as increased autoantibody production, have been noted in orchietomized B/W males treated with estrogens (Roubinian et al., 1978).

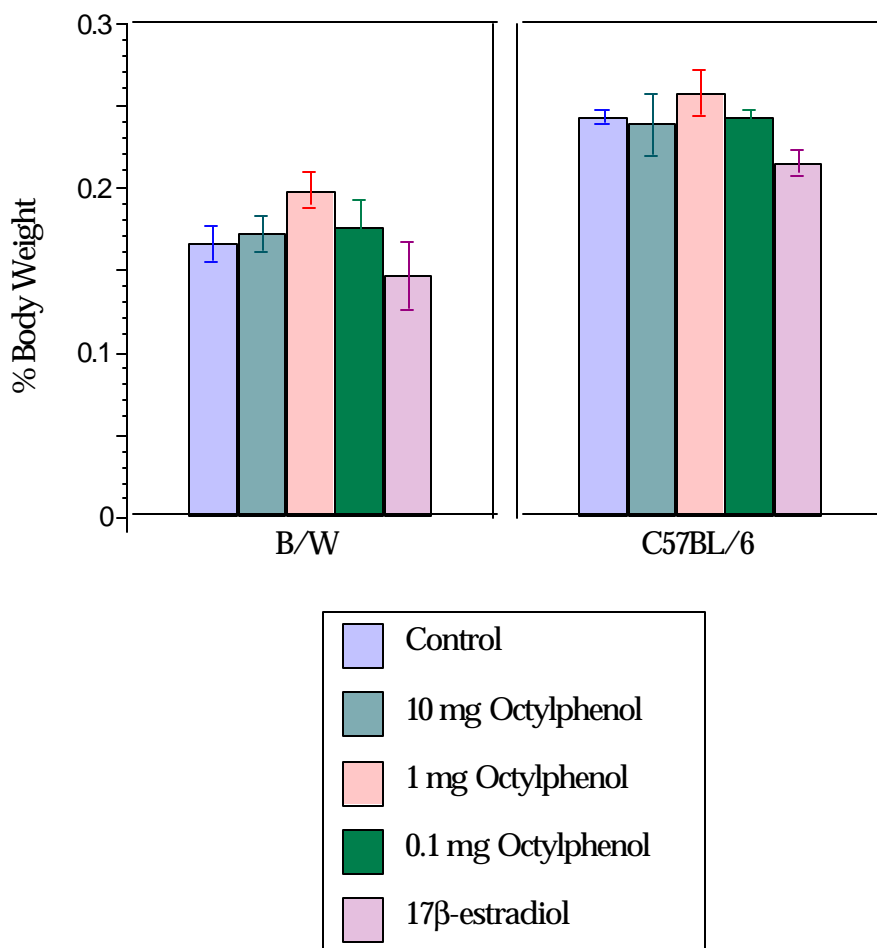
The reproductive changes that have been noted in other models when treated with a comparable dose/gram body weight of octylphenol were not seen in either the B/W or C57BL/6 mice treated with 10 mg of octylphenol. However, both the route of exposure and the model were different in the previous studies that we analyzed to determine the dose of octylphenol to be administered. In the previous studies, octylphenol was administered by subcutaneous injection to rats. The route of exposure is very important when testing the effects of any chemical. Subcutaneous injection and oral dosing lead to two different pathways of exposure in the body. Subcutaneous injection leads to a direct absorption into circulation and eventual metabolism in the liver and kidneys. Oral dosing leads to possible inactivation in the high pH of the stomach, followed by absorption from the digestive tract and direct transport to the liver for detoxification and metabolism prior to entry into circulation. The different route of exposure may account for the different effects on the reproductive tissue. The differences between metabolism in a rat and in a mouse may also account for the different effects noted.

The doses of octylphenol tested did not mimic the estrogenic effects seen with the 17 $\beta$ -estradiol treatment, and the 17 $\beta$ -estradiol treatment did not exhibit estrogenic effects to the extent which have been noted in other studies. Furthermore, 17 $\beta$ -estradiol treatment had differential effects between the B/W and C57BL/6 strains. A decrease in thymocyte and bone marrow cellularity was seen in the B/W males, but not in the C57BL/6 males, and a decrease in the % body weight of the seminal vesicles was noted in the C57BL/6 males, but not in the B/W males. It may be that the reproductive tract of the C57BL/6 males is more sensitive to estrogenic treatment than the immune system, and the immune system of the B/W males is more sensitive to estrogenic treatment than the reproductive tract. However, estrogen treatment has been shown to cause thymic atrophy in the C57BL/6 model and has been shown to cause a decrease in

bone marrow cellularity, which was not seen in the C57BL/6 males. Also, the decrease in thymocyte and bone marrow cellularity in  $17\beta$ -estradiol treated B/W males was not accompanied by a decrease in the proportion of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Estrogen induced thymic atrophy has been shown to result from a decrease in the CD4<sup>+</sup>CD8<sup>+</sup> thymocytes located in the thymic cortex (Scarpanti et al., 1989; Silverstone et al., 1994). Furthermore, no signs of accelerated onset of autoimmune disease were noted in the kidneys, liver, or salivary glands of  $17\beta$ -estradiol treated mice. As with the octylphenol treatment, the mice are only 3 – 3 ½ months old at the end of the treatment and may not have been old enough to see histopathological changes. In contrast, significant changes were noted in the kinetic studies of apoptosis. Estrogen treatment has not been shown to cause changes in the apoptosis of thymocytes in other studies. However, as with the changes noted from octylphenol treatment, significant changes were only noted in certain levels of 7-AAD fluorescence at isolated time. No overall changes in kinetics were noted, nor could any trends be deciphered. The route of administration, again, may account for the different results noted in this study compared to previous studies. Previous studies mainly utilized subcutaneous injection or implants. Although the dose of  $17\beta$ -estradiol was increased in these studies to account for the loss of activity due to oral dosing, it may not have been enough.

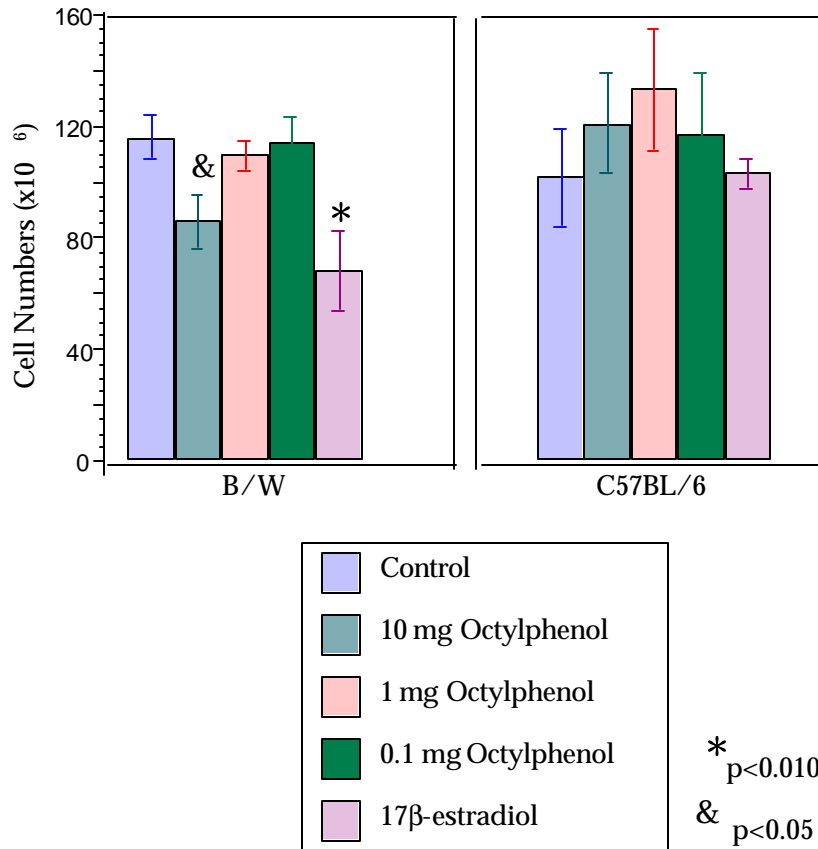
Treatment with 0.1 mg, 1 mg, or 10 mg did not affect the morphology or function of the thymus. However, octylphenol treatment and  $17\beta$ -estradiol treatment did not result in the estrogenic changes noted in other studies. A difference in the route of administration may account for the differences in the findings between our study and other studies. Therefore, further studies should be performed to determine whether subcutaneous injection of octylphenol will cause estrogenic changes in the thymus or reproductive tract in our model, and to determine whether differential estrogenic effects will be seen between the injection and feeding of octylphenol and  $17\beta$ -estradiol.

### Effect of Octylphenol Treatment on the Percent Body Weight of the Thymus in B/W and C57BL/6 Mice



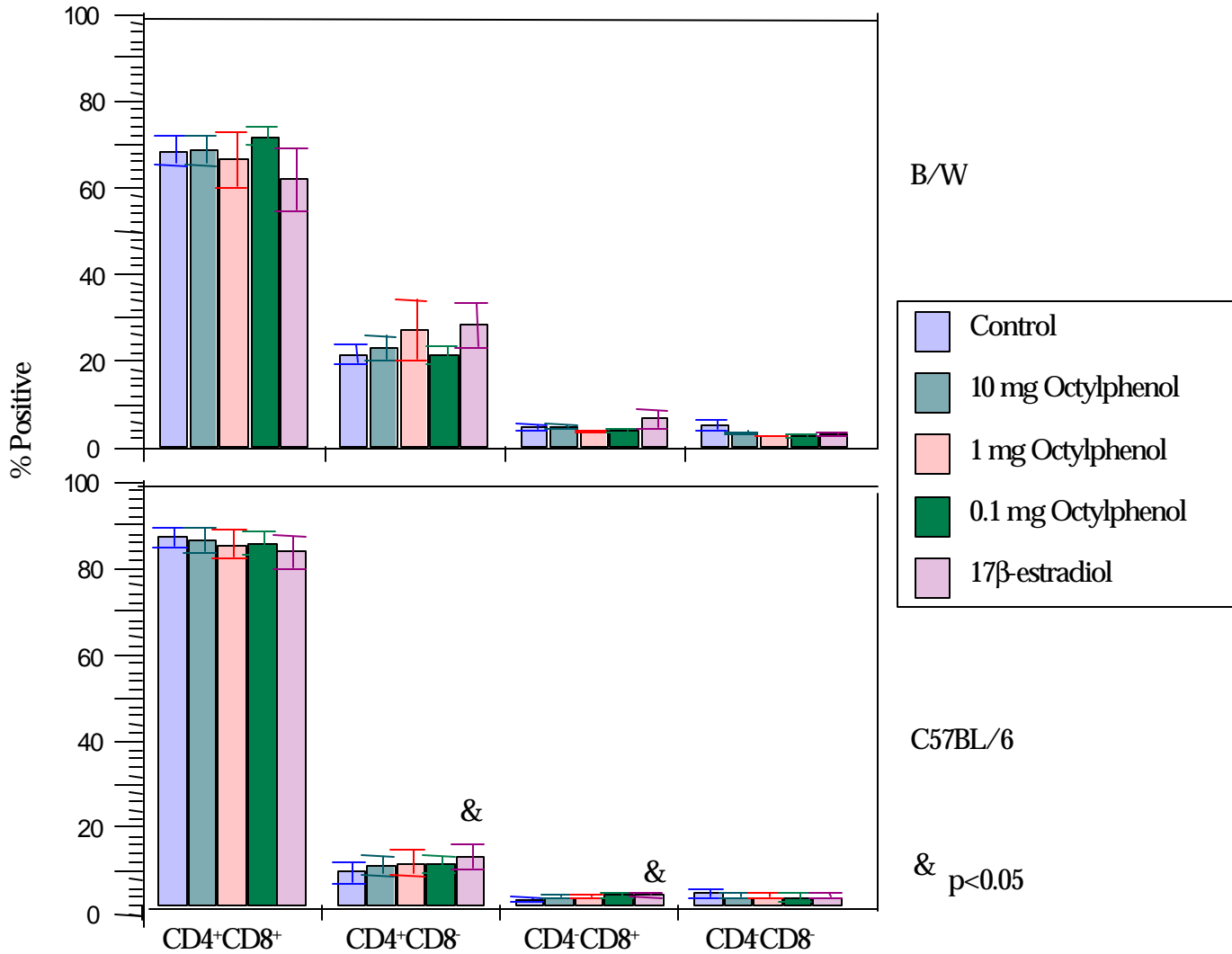
**Fig. 3.1:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment both strains were weighed and the thymus was removed. The thymus was weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

### Effect of Octylphenol Treatment on Thymocyte Cellularity in B/W and C57BL/6 Mice



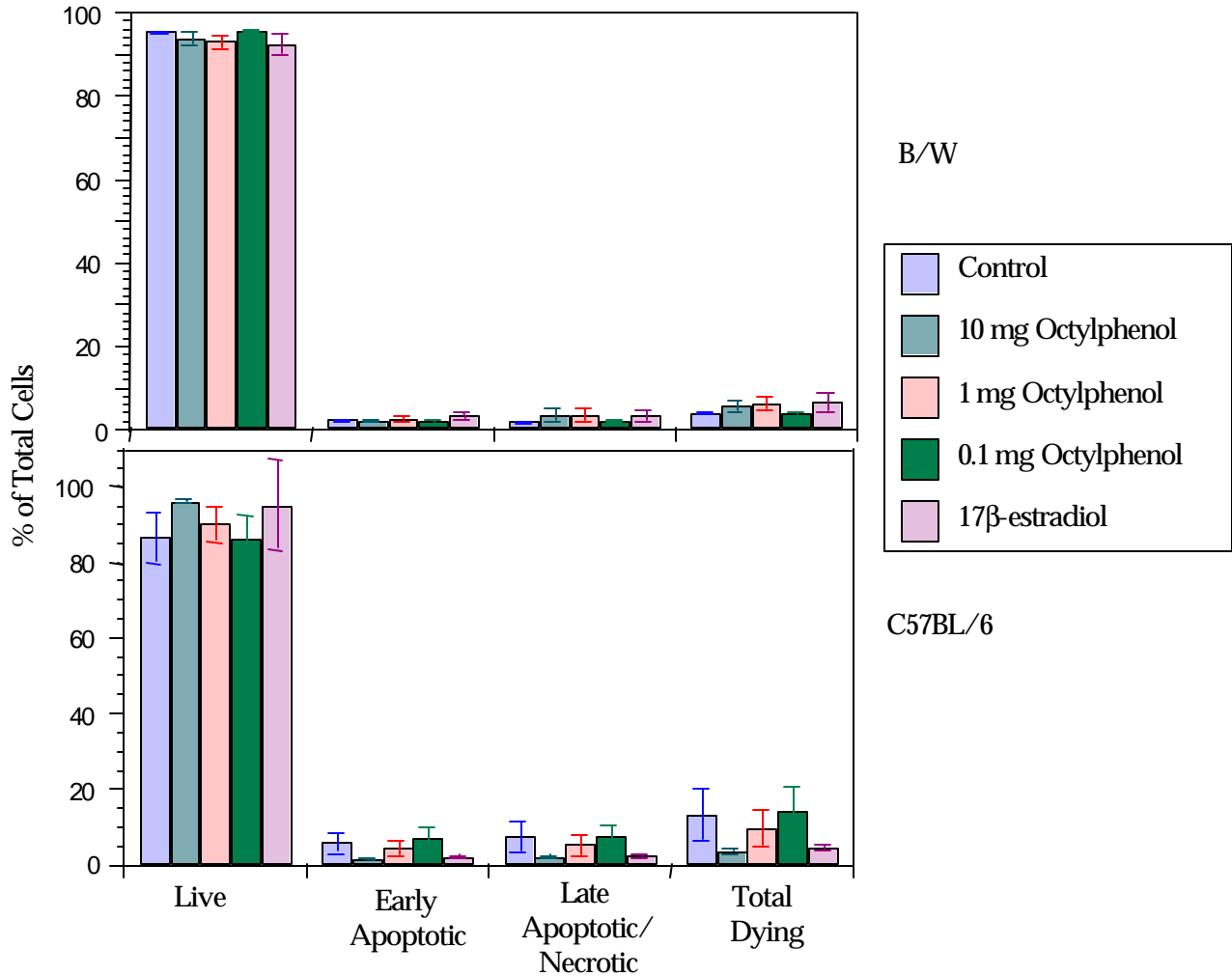
**Fig. 3.2:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment both strains had the thymus removed. The thymocytes were isolated and counted.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

### Effects of Octylphenol Treatment on the Proportion of Mature and Immature Thymocytes in B/W and C57BL/6 Mice



**Fig. 3.3:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu\text{g}$  17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymocytes were isolated from both strains and stained with PE-conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8a antibodies or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

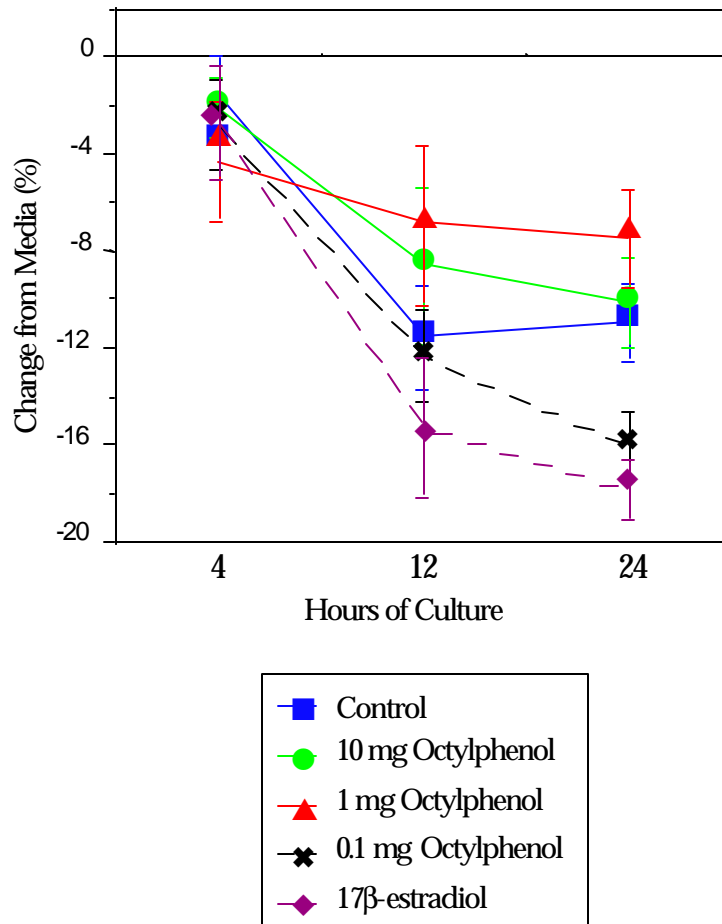
### Effect of Octylphenol Treatment on Fresh Apoptosis of Thymocytes in B/W and C57BL/6 Mice



**Fig. 3.4:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are presented as the percentage of cells that are 7-AAD<sup>dull</sup> (live), 7-AAD<sup>intermediate</sup> (early apoptotic), and 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying).  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

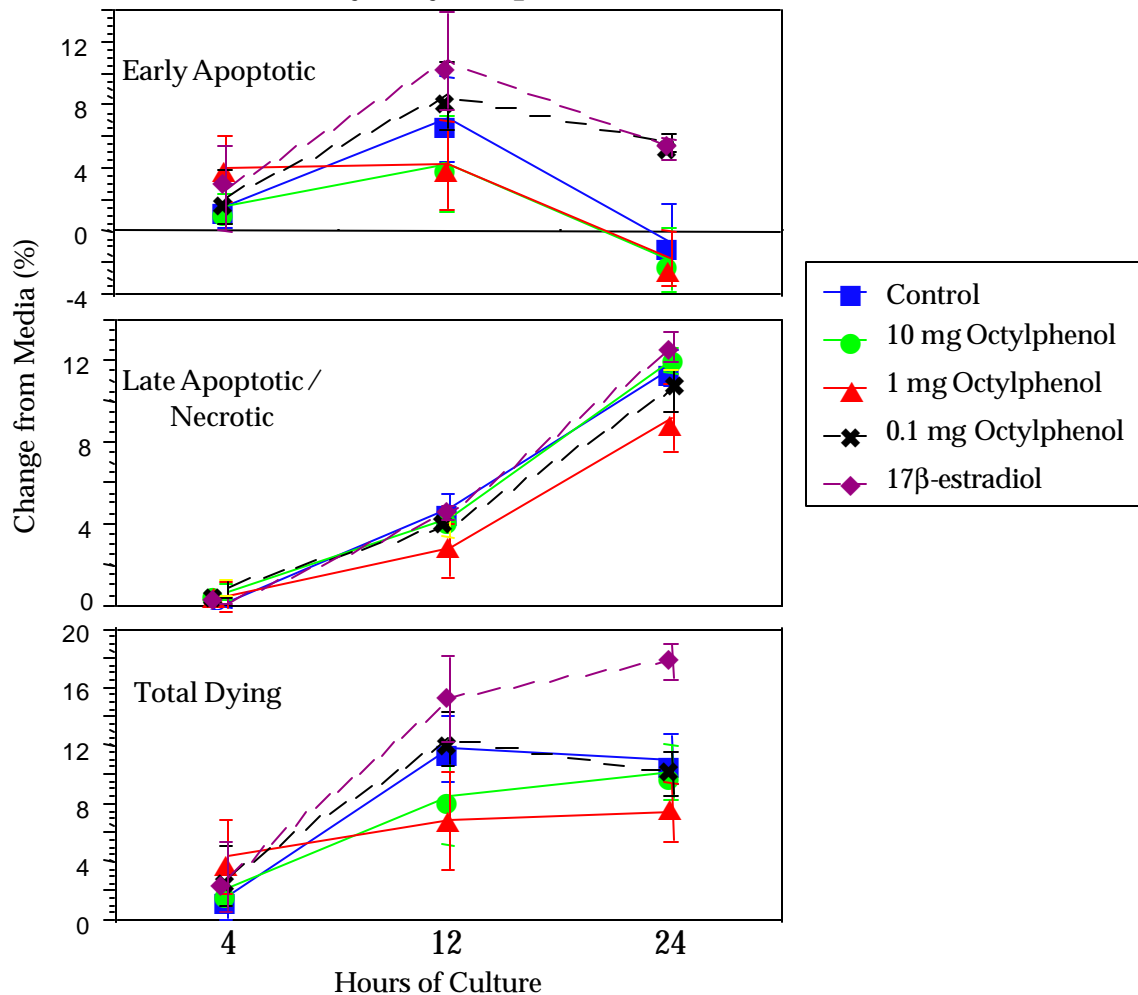


**Effect of Octylphenol Treatment in B/W Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon Anti-CD3 Stimulation**



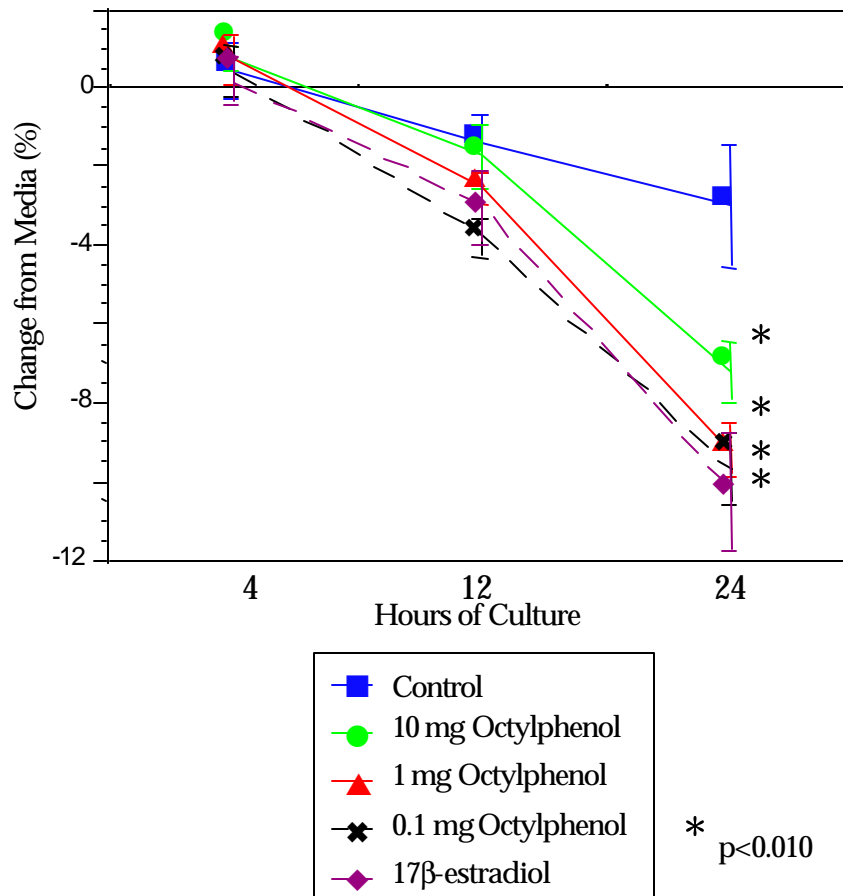
**Fig. 3.5:** Eight to ten-week old B/W males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the anti-CD3 stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=4 for 0.1 mg octylphenol at 24 hours, n=5 for 17 $\beta$ -estradiol at 4 and 24 hours, n=6 for all other groups and time points)

### Effect of Octylphenol Treatment in B/W Mice on the Apoptosis and Necrosis of Thymocytes upon Anti-CD3 Stimulation



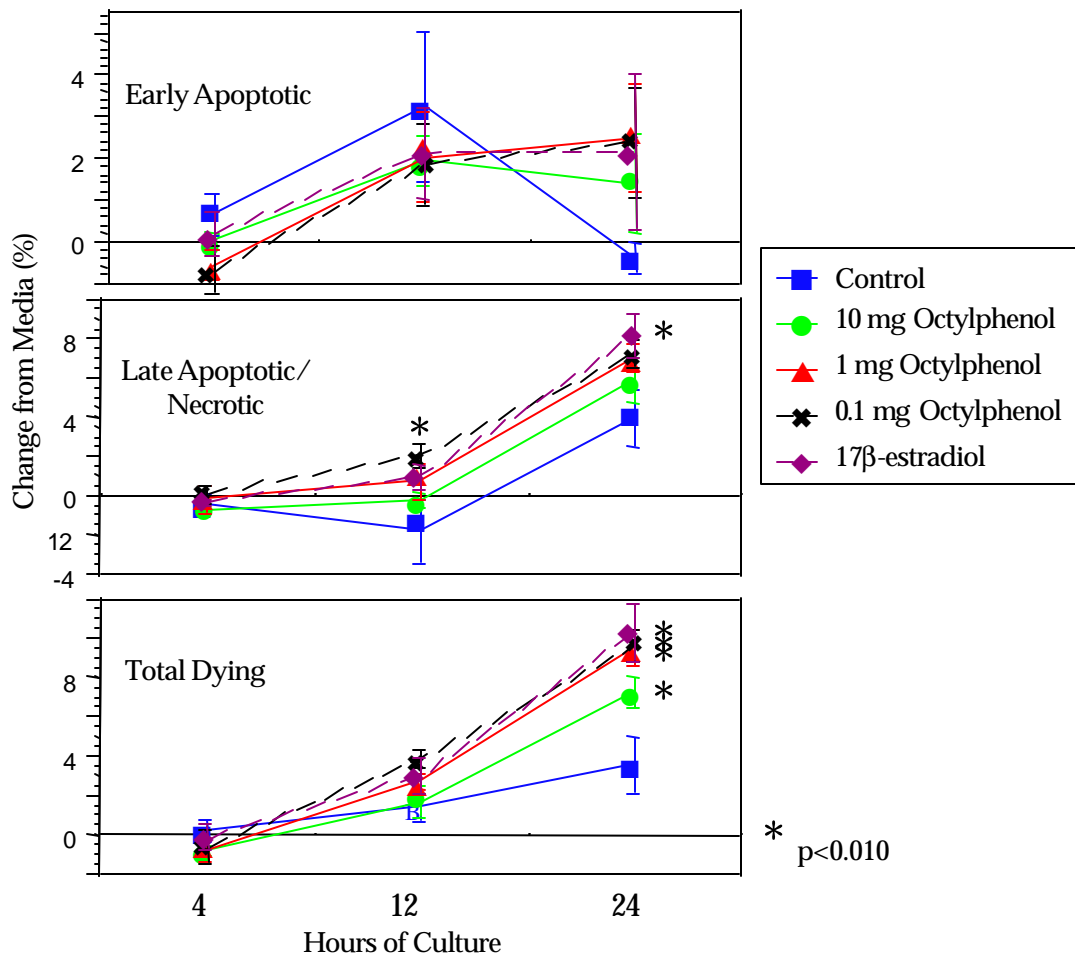
**Fig. 3.6:** Eight to ten-week old B/W males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the anti-CD3 stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=4 for 0.1 mg octylphenol at 24 hours, n=5 for 17 $\beta$ -estradiol at 4 and 24 hours, n=6 for all other groups and time points)

**Effect of Octylphenol Treatment in C57BL/6 Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon Anti-CD3 Stimulation**



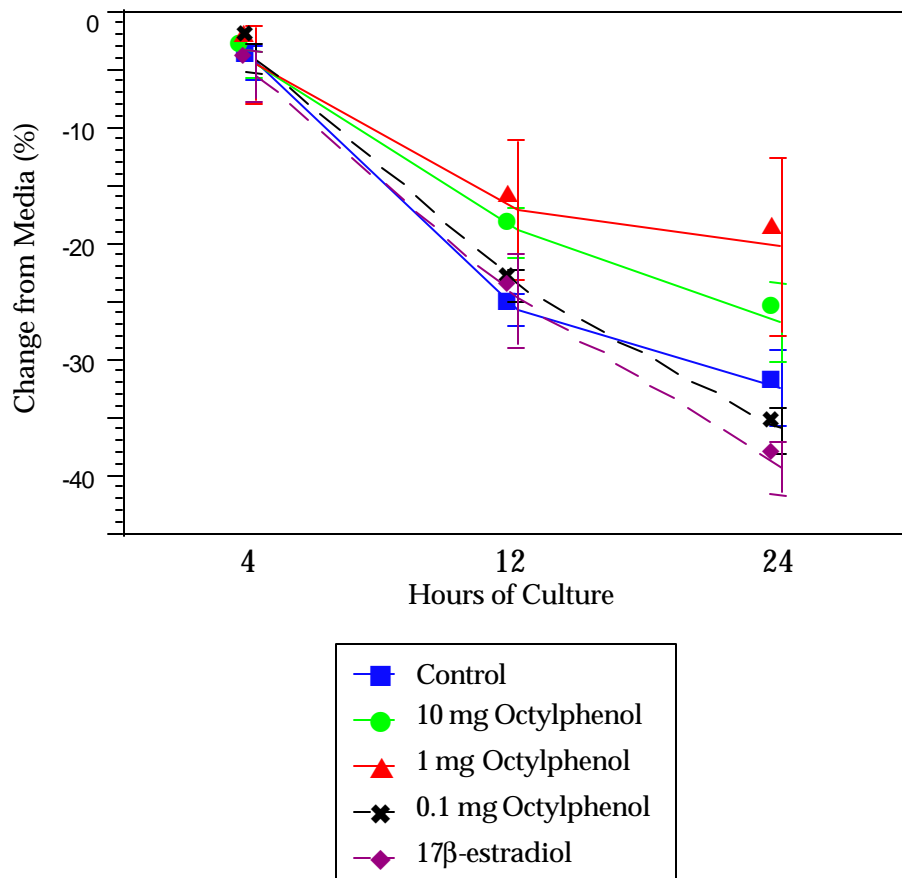
**Fig. 3.7:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 μg 17β-estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the anti-CD3 stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant (n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17β-estradiol)

### Effect of Octylphenol Treatment in C57BL/6 Mice on the Apoptosis and Necrosis of Thymocytes upon Anti-CD3 Stimulation



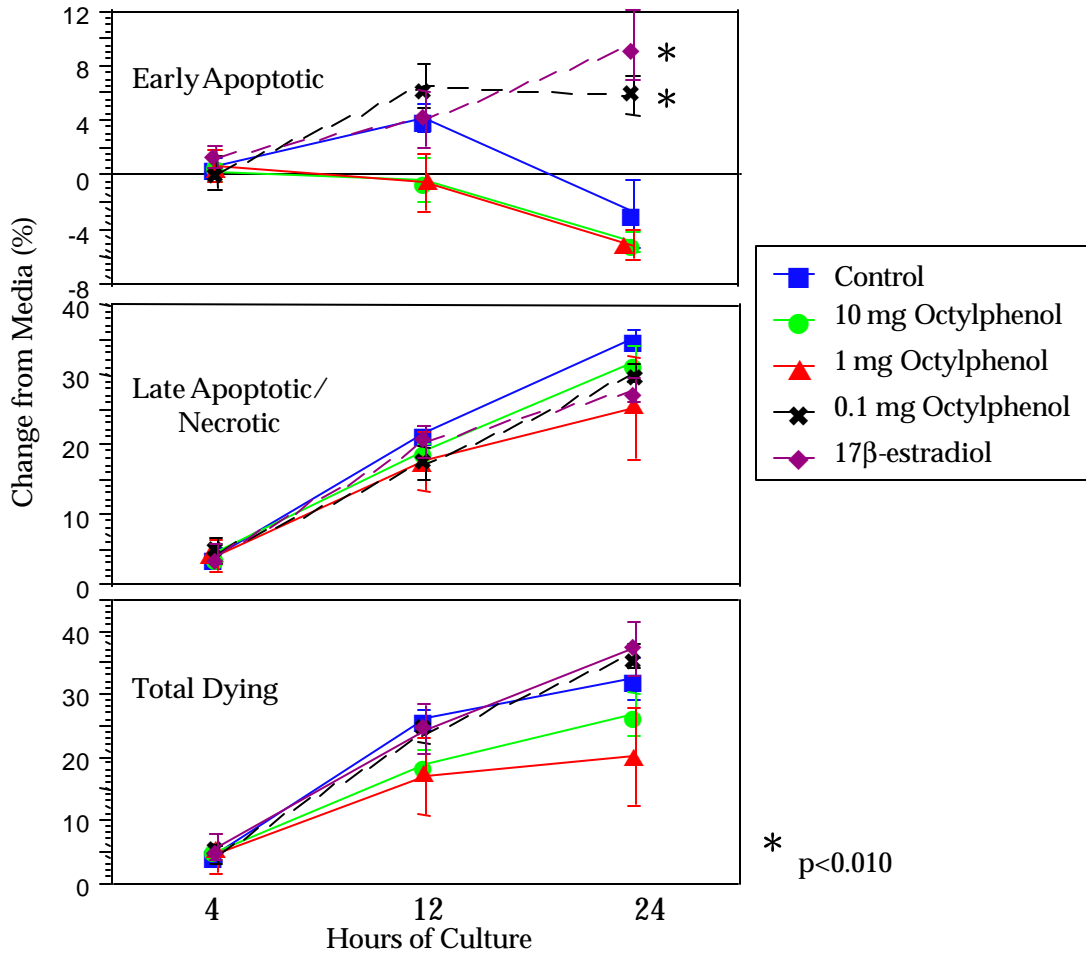
**Fig. 3.8:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the anti-CD3 stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol)

**Effect of Octylphenol Treatment in B/W Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon PMA+I Stimulation**



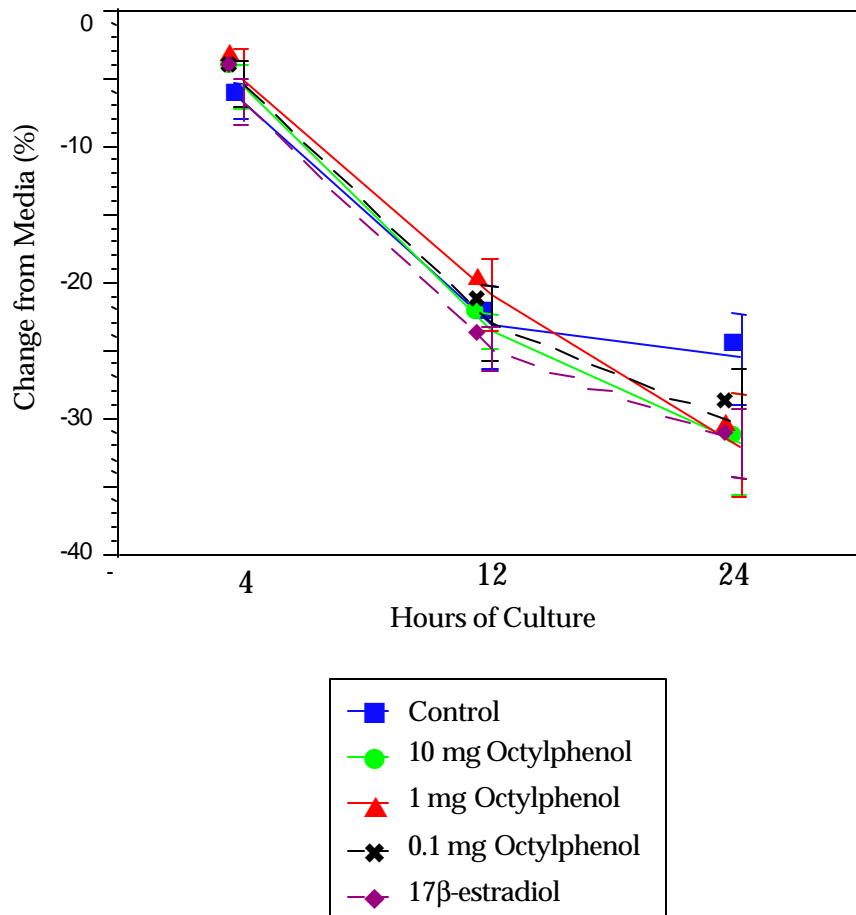
**Fig. 3.9:** Eight to ten-week old B/W males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for 17 $\beta$ -estradiol at 24 hours; n=4 for control, 0.1 mg octylphenol, 1 mg octylphenol, and 10 mg octylphenol at 24 hours; n=5 for 17 $\beta$ -estradiol at 4 hours; n=6 for all other groups and time points)

### Effect of Octylphenol Treatment in B/W Mice on the Apoptosis and Necrosis of Thymocytes upon PMA+I Stimulation



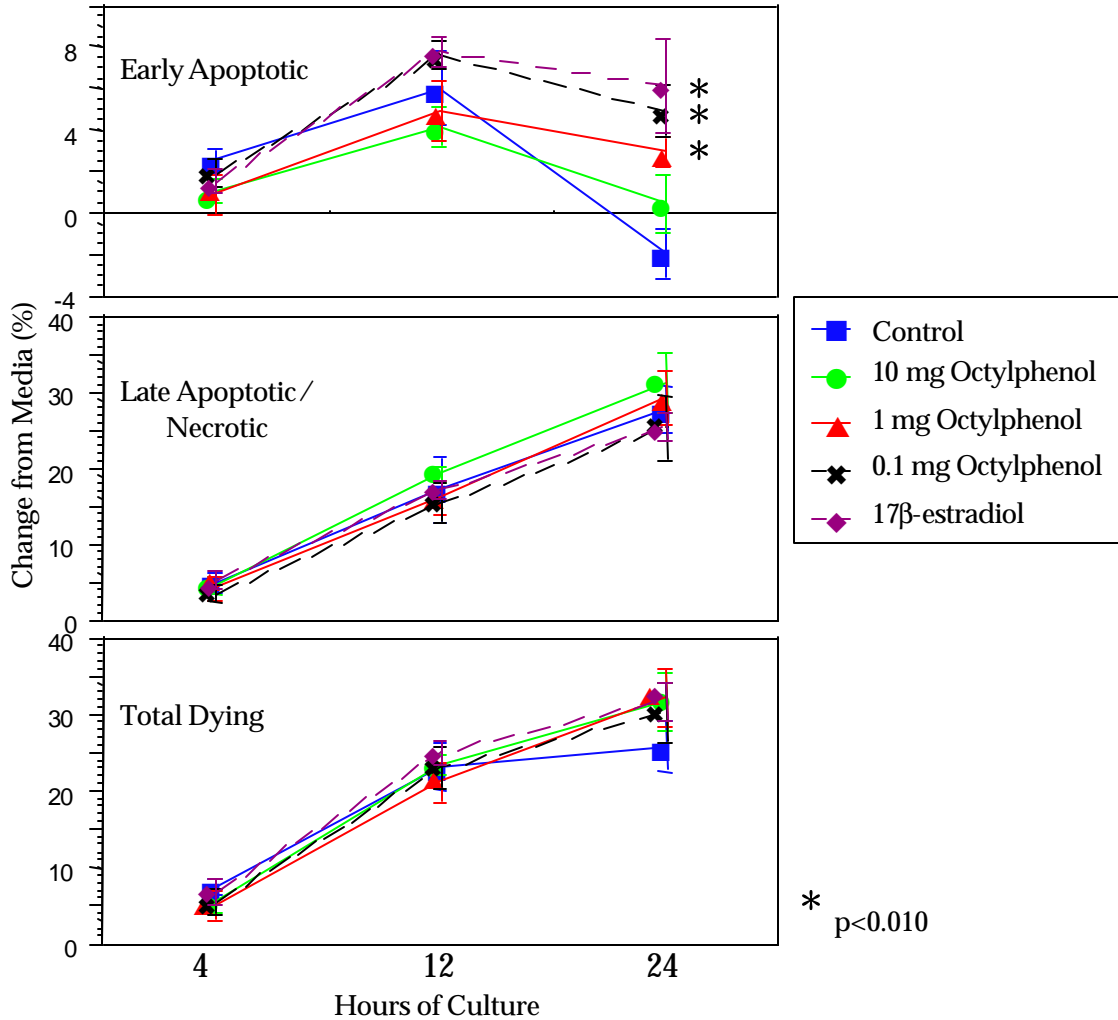
**Fig. 3.10:** Eight to ten-week old B/W males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for 17 $\beta$ -estradiol at 24 hours; n=4 for control, 0.1 mg octylphenol, 1 mg octylphenol, and 10 mg octylphenol at 24 hours; n=5 for 17 $\beta$ -estradiol at 4 hours; n=6 for all other groups and time points)

**Effect of Octylphenol Treatment in C57BL/6 Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon PMA+I Stimulation**



**Fig. 3.11:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol)

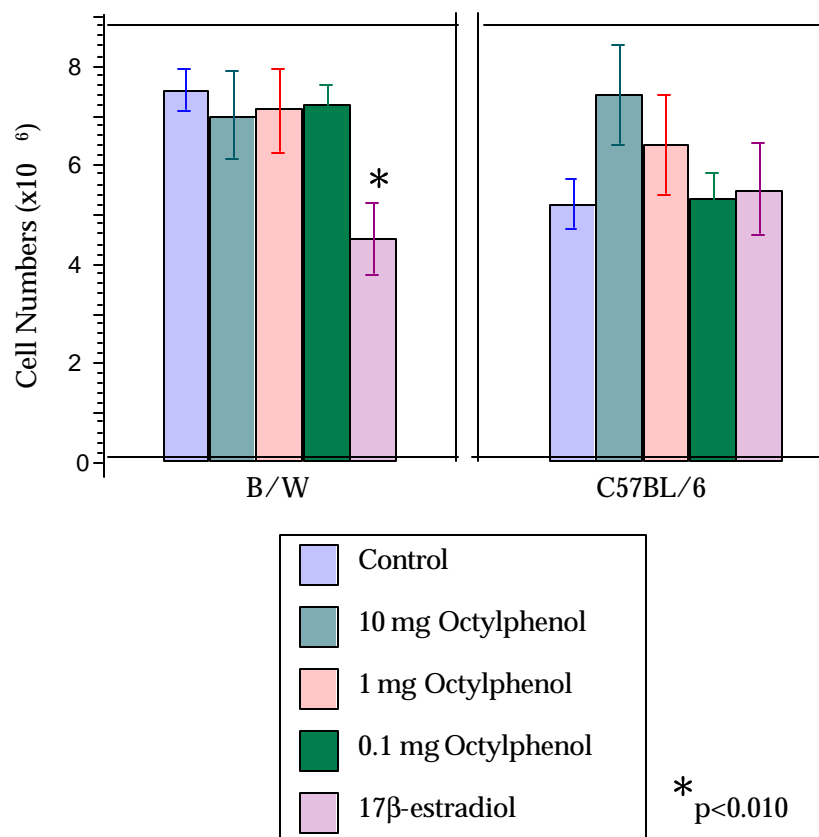
### Effect of Octylphenol Treatment in C57BL/6 Mice on the Apoptosis and Necrosis of Thymocytes upon PMA+I Stimulation



**Fig. 3.12:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol)

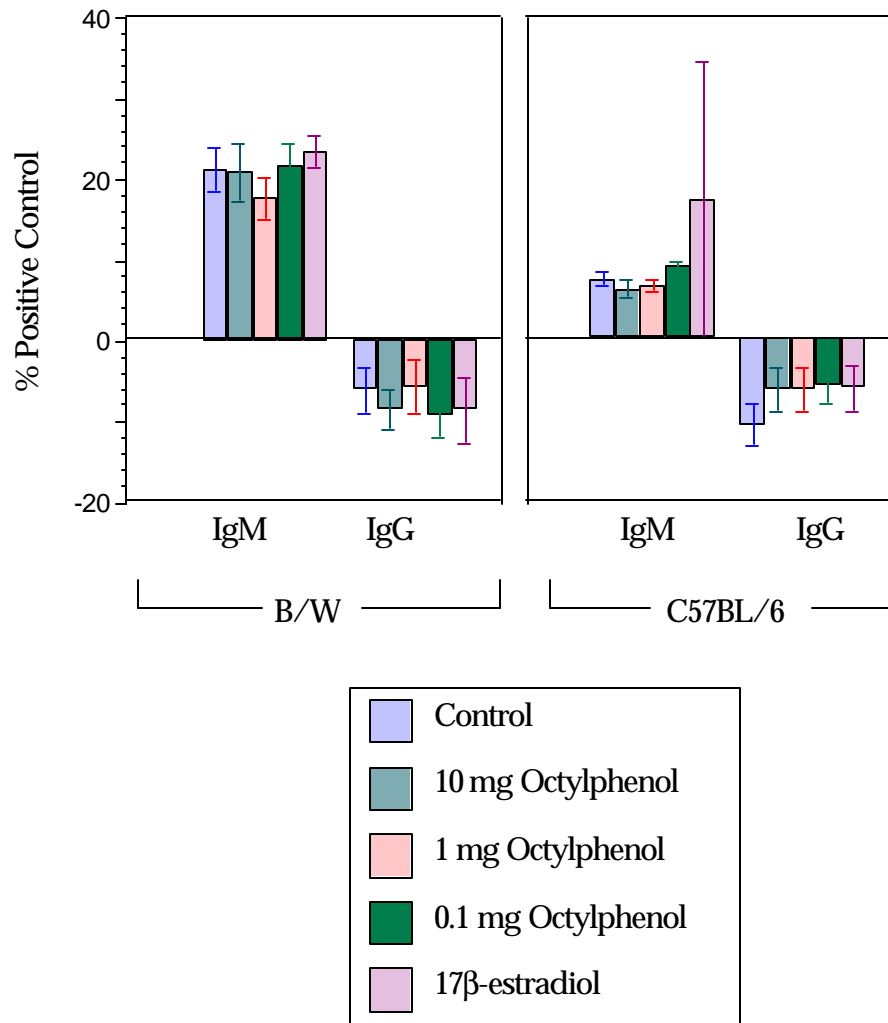


### Effect of Octylphenol Treatment on the Cellularity of the Bone Marrow of B/W and C57BL/6 Mice



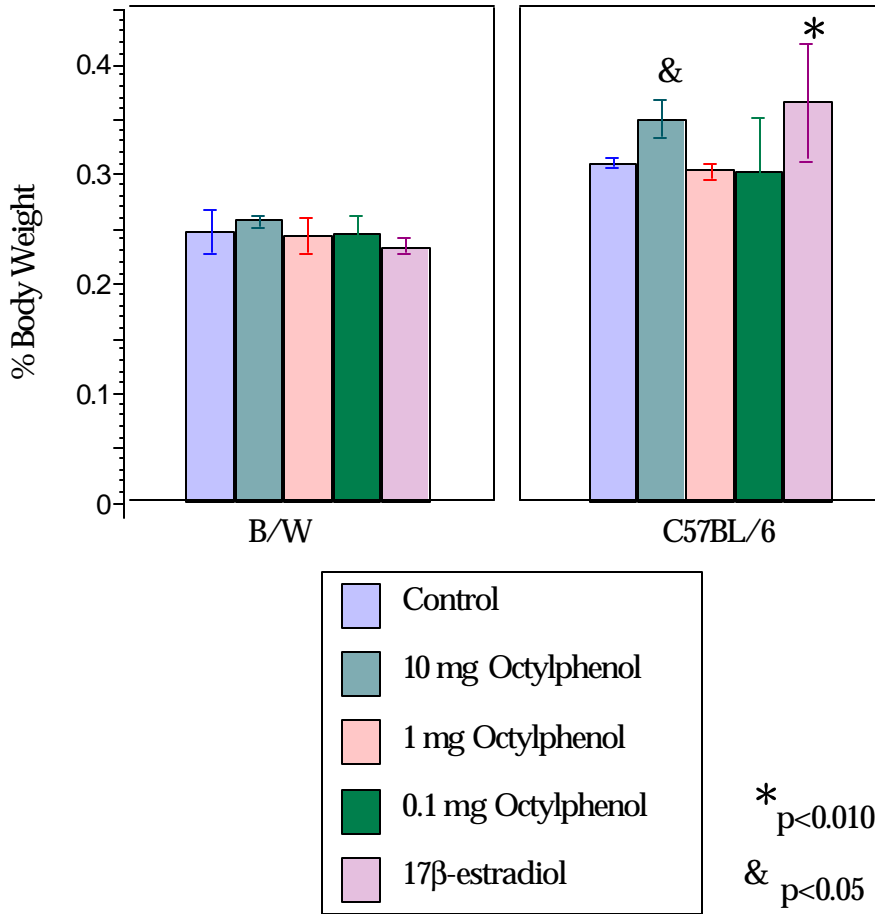
**Fig. 3.13:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the bone marrow was washed from the left femur of both strains, the red blood cells were removed, and the remaining cells were counted.  $p \leq 0.010$  was considered significant. (C57BL/6: n=4 for control, n=4 for 10 mg octylphenol, n=4 for 1 mg octylphenol, n=4 for 0.1 mg octylphenol, n=4 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

### Effect of Octylphenol Treatment on the Level of Anti-dsDNA Autoantibodies in the Serum of B/W and C57BL/6 Mice



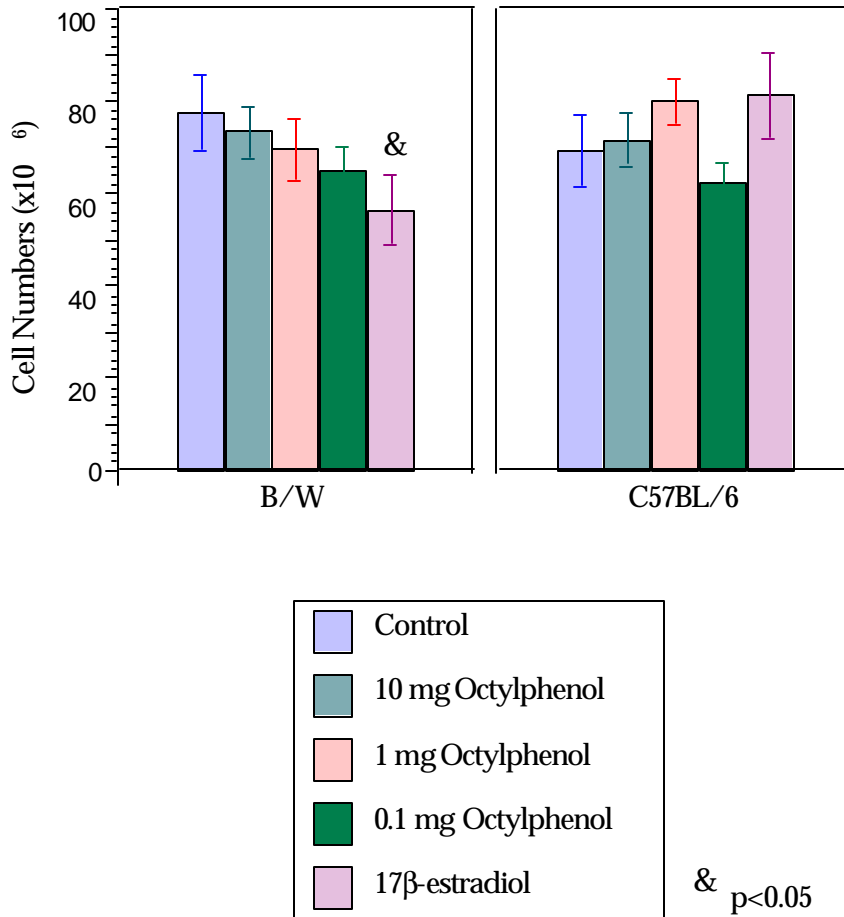
**Fig. 3.14:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment both strains were bled retro-orbitally and the serum removed. ELISAs were performed to determine the level of IgM and IgG anti-dsDNA autoantibodies in the serum. The data are presented as the percentage of the positive control serum to control for plate to plate variation.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

### Effect of Octylphenol Treatment on the Percent Body Weight of the Spleen in B/W and C57BL/6 Mice



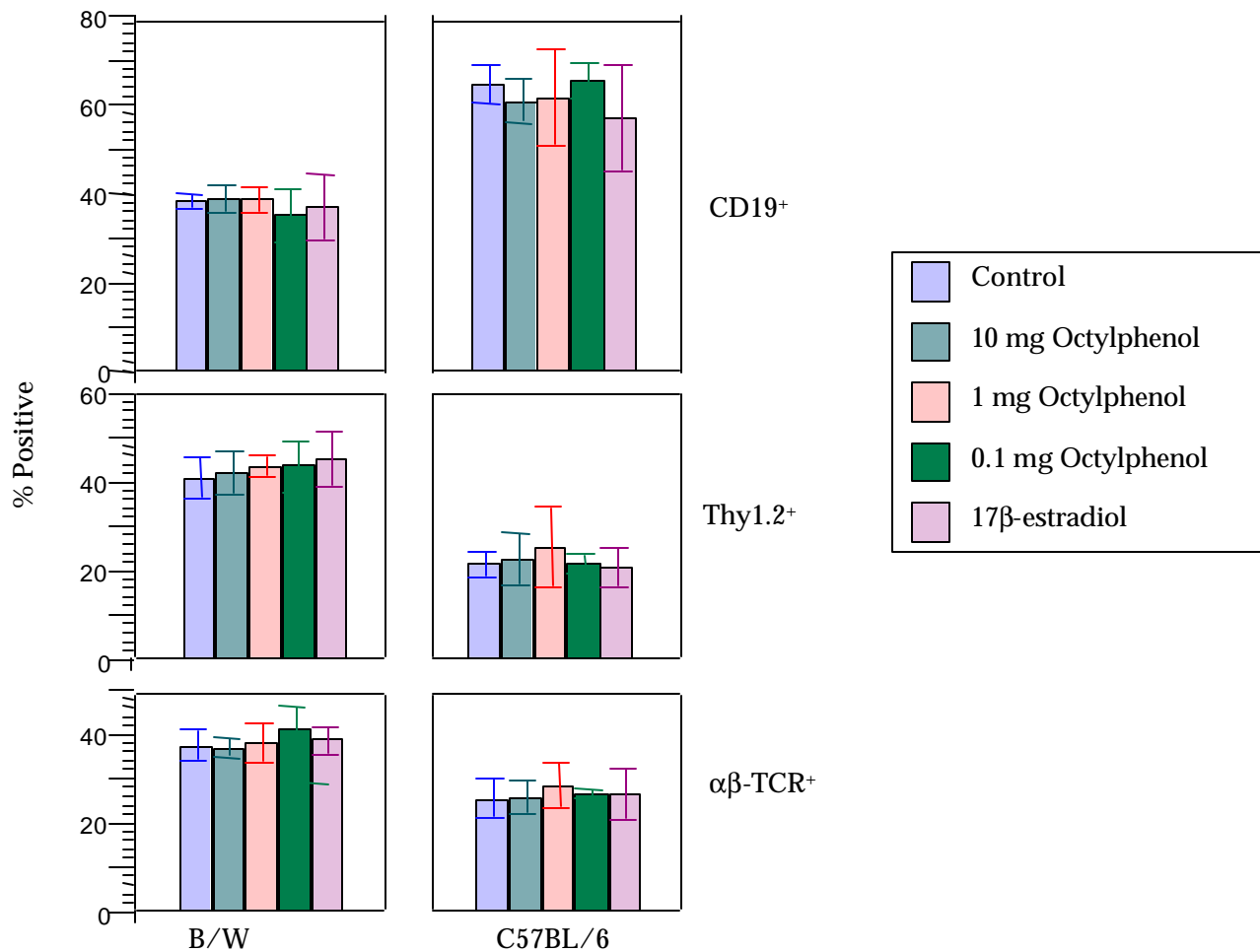
**Fig. 3.15:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9 μg 17β-estradiol, or an ethanol/oil control. Three days after the last treatment both strains were weighed and the spleen was removed. The spleen was weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17β-estradiol; B/W: n=6 for all groups)

### Effect of Octylphenol Treatment on the Leukocyte Cellularity of the Spleen in B/W and C57BL/6 Mice



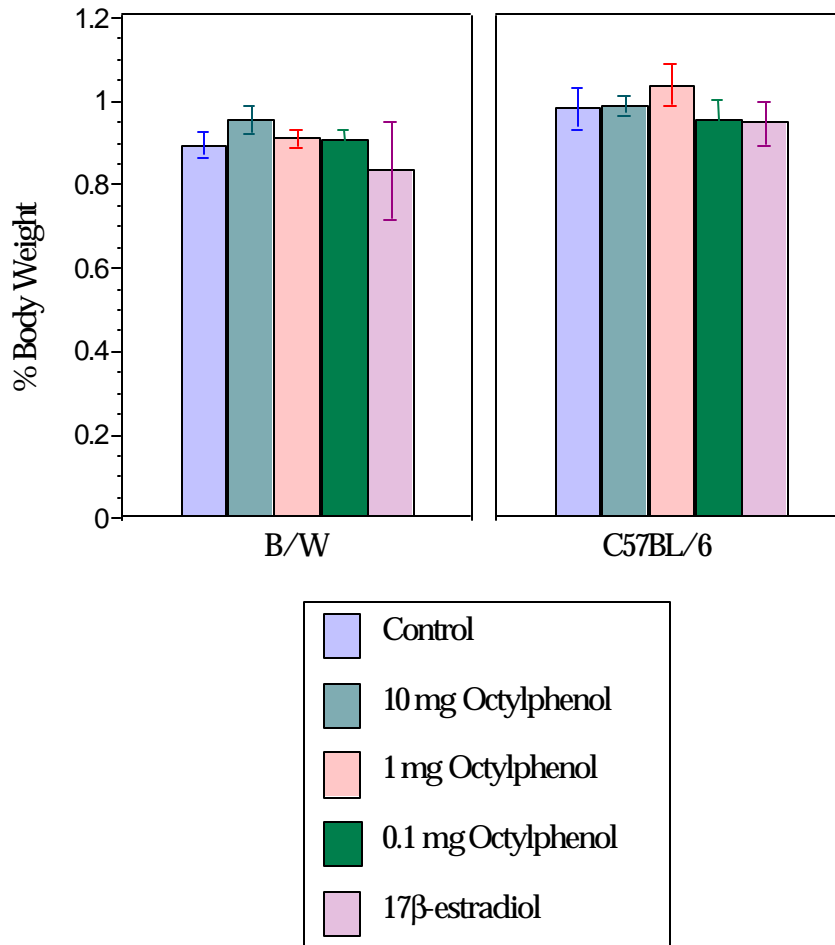
**Fig. 3.16:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the spleen was removed from both strains. The leukocytes were isolated and counted.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

### Effect of Octylphenol Treatment on B and T Cell Markers of Splenic Leukocytes in B/W and C57BL/6 Mice



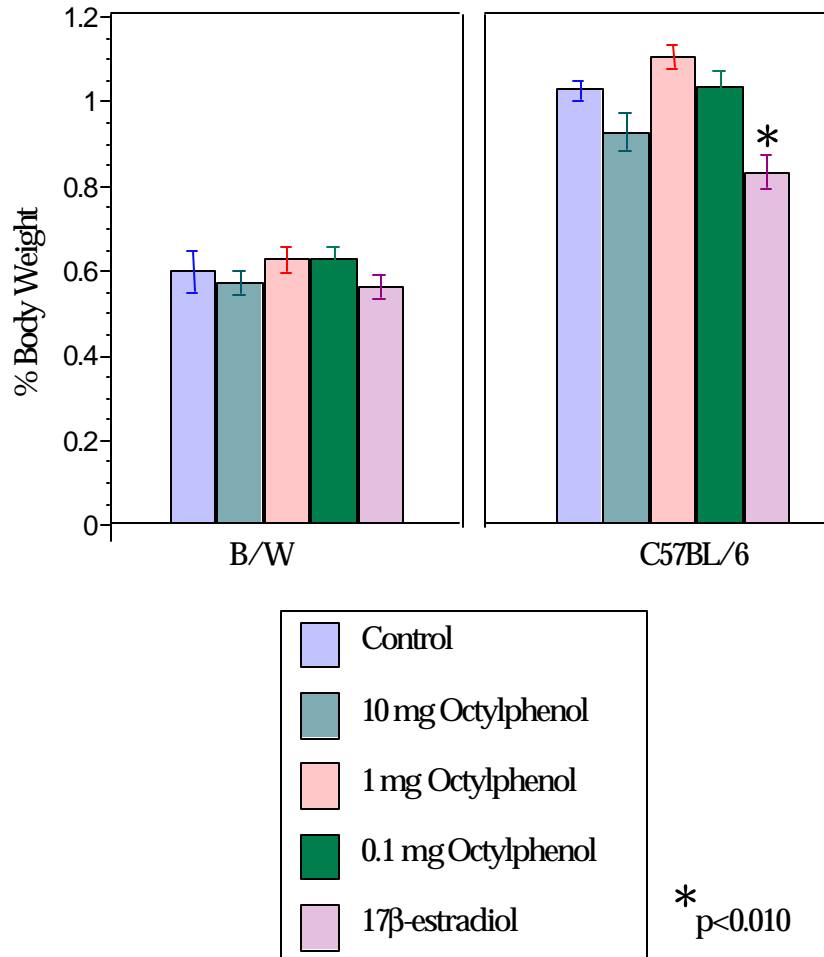
**Fig. 3.17:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment the spleen was removed from both strains. The leukocytes were isolated from the spleen and stained with PE-conjugated anti-CD19 antibodies and FITC-conjugated anti-Thy1.2 antibodies, cy-chrome-conjugated anti- $\alpha\beta$ TCR antibodies, or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6:  $n=6$  for control,  $n=7$  for 10 mg octylphenol,  $n=6$  for 1 mg octylphenol,  $n=5$  for 0.1 mg octylphenol,  $n=7$  for 17 $\beta$ -estradiol -  $\alpha\beta$ TCR;  $n=4$  for all groups - Thy1.2 and CD19; B/W:  $n=6$  for all groups -  $\alpha\beta$ TCR and Thy1.2;  $n=4$  for all groups - CD19)

### Effect of Octylphenol Treatment on the Percent Body Weight of the Testis in B/W and C57BL/6 Mice



**Fig. 3.18:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment both strains were weighed and the testis were removed. The testis were weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=6 for all groups)

### Effect of Octylphenol Treatment on the Percent Body Weight of the Seminal Vesicles in B/W and C57BL/6 Mice



**Fig. 3.19:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with 0.1 mg, 1 mg, or 10 mg of octylphenol, 6 or 9  $\mu$ g 17 $\beta$ -estradiol, or an ethanol/oil control. Three days after the last treatment both strains were weighed and the seminal vesicles were removed. The seminal vesicles were weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6: n=6 for control, n=7 for 10 mg octylphenol, n=6 for 1 mg octylphenol, n=5 for 0.1 mg octylphenol, n=7 for 17 $\beta$ -estradiol; B/W: n=4 for all groups)

## Chapter 4

### **Route of administration studies to compare the effects of oral and subcutaneous administration of octylphenol and 17 $\beta$ -estradiol in C57BL/6 and B/W Mice**

#### **a.) Introduction**

The dose range studies, which looked at the effects of octylphenol on the thymus of adult male C57BL/6 and B/W mice, exhibited no immunological or reproductive effects on the at oral doses of 0.1 mg, 1 mg, or 10 mg. Two main differences existed between the model in the preliminary studies and that of the dose range experiments. First, both models utilized adult mice, but the mice were 3 months older in the preliminary studies. Second, the preliminary studies utilized orchietomized mice, whereas the dose range studies utilized reproductively intact mice to study a more relevant model. Either change in the model could have caused the different responses seen in the 10 mg treatment of octylphenol.

However, to complicate matters, 17 $\beta$ -estradiol also did not exhibit the estrogenic effects that have been seen in other studies. The 17 $\beta$ -estradiol treatment did not induce the thymic atrophy expected from findings in previous studies and a decrease in the weights of the seminal vesicles was only seen in the C57BL/6 mice, not in the B/W mice. Furthermore, a decrease in bone marrow cellularity was noted in the 17 $\beta$ -estradiol treated B/W mice, but not in the C57BL/6 mice. In the majority of previous studies, 17 $\beta$ -estradiol was administered either by subcutaneous injection or subcutaneous implant (Seaman and Gindhart, 1979; Ansar Ahmed et al., 1985a; Ansar Ahmed et al., 1989; Screpanti et al., 1989; Ansar Ahmed and Verthelyi, 1993; Silverstone et al., 1994; Verthelyi and Ansar Ahmed, 1994, 1997, 1998; Holland and Roy, 1995; Olsen and Kovacs, 1996). Therefore, choosing a dose that induces thymic atrophy upon oral administration was not obvious from the literature. The dose of 17 $\beta$ -estradiol was based on previous studies that examined the induction of thymic atrophy by subcutaneous injection (Ansar Ahmed et al., 1985b). In this study, the dose which has been shown to induce thymic atrophy after short-term administration of 17 $\beta$ -estradiol by subcutaneous injection was increase by 3X and given orally in the previous studies.



The route of exposure is a likely explanation for the differences in the findings of our study compared to those in previous studies. Previous studies on the reproductive changes caused by octylphenol have utilized subcutaneous injections, and most studies on the estrogenic effects of 17 $\beta$ -estradiol have also utilized subcutaneous administration of an injection or an implant. Subcutaneous exposure, especially with injections, leads to a high dose absorbed directly into circulation, which is eventually taken to the liver for metabolism and detoxification. Upon oral dosing a compound must enter the high pH of the stomach, and then lipid soluble compounds are absorbed into circulation in the small intestines, which has a portal directly to the liver for metabolism and detoxification. The different routes of exposure can directly effect the potency to the compounds tested. Therefore, in order to determine whether octylphenol has estrogenic effects on the thymus or on the reproductive tissue as seen in other studies, octylphenol and 17 $\beta$ -estradiol were subcutaneously injected in C57BL/6 or B/W adult males. The effects on the morphology and function of the thymus were noted, along with reproductive changes. Furthermore, to determine the importance of the route of administration for estrogenic activity of octylphenol and 17 $\beta$ -estradiol, oral feeding of octylphenol and 17 $\beta$ -estradiol will be compared to subcutaneous injection in the parameters measured.

### **b.) Specific Aims**

I.) The primary aim of this study is to determine whether the subcutaneous injection of octylphenol at 10 mg will affect the morphology and function of the thymus of C57BL/6 and B/W adult males and will cause reproductive changes. The following were examined:

a.) Morphology of Primary Lymphoid Organs:

- ❖ Thymus weight and cellularity
- ❖ Proportion of immature and mature thymocytes.
- ❖ Bone marrow cellularity to determine whether a decrease in thymic cellularity is due to a decrease in precursor cells from the bone marrow

b.) Function of the thymus:

- ❖ Proportion of immature and mature thymocytes to determines whether proper development of T cells is occurring
- ❖ Apoptosis of unstimulated and stimulated thymocytes to determine whether there is proper signaling for apoptosis

c.) Autoimmune Parameters:

- ❖ Autoantibody production
- ❖ Histopathologic examination of the kidneys, liver and salivary glands to determine whether proper selection is occurring

d.) Morphology of Secondary Lymphoid Organs:

- ❖ Splenic weight and leukocyte cellularity
- ❖ T cell/B cell numbers to determine any alteration in the migration of mature T cells out of the thymus into the spleen

e.) Reproductive changes: testis and seminal vesicle weights

II) The secondary aim of this study is to determine whether the route of exposure of octylphenol and 17 $\beta$ -estradiol is important in the estrogenic effects they have on the thymus and reproductive tract of C57BL/6 and B/W adult males. Therefore, octylphenol and 17 $\beta$ -estradiol will be administered by feeding, as well as by subcutaneous injection, and the results from the above parameters will be compared

**c.) Materials and Methods**

**Mice:** Eight-to-ten week old C57BL/6 males were purchased from Charles River (Wilmington, MA) and eight-to-ten week old B/W males were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed, fed, and watered as described in Chapter 2.

**Octylphenol and 17 $\beta$ -estradiol treatment:** One week after arrival the B/W and C57BL/6 males randomly divided into treatment groups, selected mice were either fed 10 mg of octylphenol, subcutaneously injected with 10 mg of octylphenol, fed 15  $\mu$ g of 17 $\beta$ -estradiol, subcutaneously injected with 15  $\mu$ g of 17 $\beta$ -estradiol, or injected with an ethanol/oil control three times a week for three weeks. Octylphenol (Sigma, St. Louis, MO) was dissolved in ethanol to make a stock concentration of 20 mg/30  $\mu$ l. A 1:2 dilution in autoclaved corn oil (ICN, Aurora, OH) of the stock solution gave a 10 mg/30  $\mu$ l solution for feeding and injection. A solution of 17 $\beta$ -estradiol (Sigma) in ethanol at a concentration of 30  $\mu$ g/30  $\mu$ l was made. A 1:2 dilution in corn oil of the 17 $\beta$ -estradiol solution gave a 15  $\mu$ g/30  $\mu$ l solution for feeding and injection. Negative control mice were injected with equal parts ethanol and corn oil. The mice were either fed or subcutaneously injected with 30  $\mu$ l of one of the treatments three times a week for three weeks. The subcutaneous injections were rotated between the left and right abdominal area and the back. All solutions were made fresh each day of

treatment. Three days after the last treatment the mice were euthanized by cervical dislocation as approved by the Virginia Polytechnic Institute and State University Animal Care Committee.

**Serum collection and detection of dsDNA antibodies:** Mice were bled retro-orbitally prior to euthanization. Serum was collected and kept frozen until tested for dsDNA antibodies. The dsDNA antibodies were detected in the serum by standard ELISA techniques as described previously in Chapter 3. The absorbance of the test sera was analyzed as the percent of the positive controls per plate [(O.D.-negative control/positive control)\*100].

**Lymphoid tissue collection and processing:** The mice were weighed and the spleen, thymus, and bone marrow were removed and processed as described previously in Chapter 3. The body weights taken prior to euthanization were utilized in the percent body weights ratios reported for the spleen and thymus. The number of lymphocytes isolated from thymus and the number of leukocytes isolated from the spleen and bone marrow was noted, and all cells were adjusted to a density of  $5 \times 10^6$  cells/ml in complete RPMI and kept at 4°C until utilized in assays.

**Non-lymphoid tissue collection and processing:** The testis, seminal vesicles, kidneys, liver, and salivary glands were removed from each mouse and examined histopathologically as described previously in Chapter 3. In some mice, a small section of the spleen was also sent for histopathological examination. These spleens were not utilized in cellularity counts. The testis and seminal vesicles were weighed and the percent body weight was noted.

**Flow cytometric analysis of cell markers:** Thymus, spleen, and bone marrow cells were stained with appropriate monoclonal antibodies and analyzed by flow cytometry as described previously in Chapter 2. The following antibodies were utilized in these studies: fluorescein isothiocyanate (FITC)-conjugated anti-CD8a (53-6.7), anti-Thy1.2 (CD90, G7), anti-F480 (F480), anti-CD11b (Mac1, M1/70), and rat IgG<sub>2a</sub> isotype control (R35-95); phycoerythrin (PE)-conjugated anti-CD4 (RM4-5), anti-CD19 (1D3), anti-CD71 (C2), anti-GR1 (Ly-6G, RB6-8C5), and rat IgG<sub>2a</sub> isotype control (R35-95); and cy-chrome-conjugated anti- $\alpha\beta$ -TCR (H57-597), and hamster IgG isotype control (G235-2356). All monoclonal antibodies were purchased from Pharmingen (San Diego, CA).

**Stimulation and expression of Very Early Activation Antigen (CD69):** Leukocytes from the spleen were plated and stimulated as previously described

in Chapter 2. After 4 hours, the plate was centrifuged at 300 X g for 6 minutes to pellet the cells and the supernatants were removed. The cells were resuspended in 100  $\mu$ l of incomplete RPMI media and stained as in the previous section for cell markers. In most experiments the cells were stained with either PE-conjugated anti-CD69 (H1.2F3) (Pharmigen) or hamster IgG isotype control (A19-3) (Pharmigen) and analyzed by the flow cytometer for fluorescence. In some experiments, the cells were double stained with FITC-conjugated anti-CD69 and PE-conjugated anti-GR1 or FITC-conjugated hamster IgG isotype control and PE-conjugated rat IgG<sub>2a</sub> isotype control.

**Detection of apoptosis:** The percent of freshly isolated and stimulated thymocytes undergoing apoptosis was determined by 7-aminoactinomycin-D (7-AAD) fluorescence as described previously in Chapter 3. The percent of 7-AAD uptake is directly related to the extent of apoptosis. Therefore, the 7-AAD<sup>dull</sup> cells are considered live, the 7-AAD<sup>intermediate</sup> cells are considered early apoptotic, and the 7-AAD<sup>bright</sup> cells are considered late apoptotic/necrotic. The 7-AAD<sup>intermediate</sup> and 7-AAD<sup>bright</sup> are combined to determine the proportion of the total dying population (7-AAD<sup>intermediate+bright</sup>). The data are represented as the change from the media baseline of the anti-CD3 and PMA/I stimulated thymocytes in each of the 7-AAD<sup>dull</sup>, 7-AAD<sup>intermediate</sup>, 7-AAD<sup>bright</sup>, and 7-AAD<sup>intermediate+bright</sup> proportions of the population.

**Statistics:** The MIXED procedure of SAS (SAS ver. 6.12, SAS Institute, Cary, NC) was used to perform a mixed effects, repeated measures ANOVA on the 7-AAD apoptosis data. A first order autoregressive model was used to model covariance among repeated measurements from a mouse. Single degree of freedom contrasts were utilized to determine whether the injected octylphenol or 17 $\beta$ -estradiol groups differed from the injected control groups and whether the injected octylphenol or 17 $\beta$ -estradiol groups. The apoptosis data were analyzed as the change from the media baseline. All other data were analyzed utilizing the General Linear Model procedure of SAS to perform a one-way ANOVA. Single degree of freedom contrasts were utilized to determine whether the injected octylphenol or 17 $\beta$ -estradiol groups differed from the injected control groups and whether the injected octylphenol or 17 $\beta$ -estradiol groups differed from the fed octylphenol or 17 $\beta$ -estradiol groups, respectively. Because of the multiple comparisons, Bonferonni correction was used to hold the experimentwise error rate to 0.05 (comparisonwise alpha = 0.01, therefore p<0.01 was considered significant).

#### **d.) Results**

**Effect of octylphenol on the morphology of the thymus:** The morphology of the thymus was altered by subcutaneous injection of 10 mg of octylphenol in the C57BL/6 males, but not the B/W males. The thymocyte numbers were significantly decreased compared to controls by subcutaneous injection of 10 mg of octylphenol and by subcutaneous injection of 15  $\mu$ g of 17 $\beta$ -estradiol in C57BL/6 males (Fig 4.1). Furthermore, injection of 17 $\beta$ -estradiol, but not octylphenol, resulted in a significant decrease in the percent body weight of the thymus in C57BL/6 males (Fig 4.2). In the B/W males, the percent body weight of the thymus and the thymocyte numbers were significantly decreased by subcutaneous injection of 17 $\beta$ -estradiol, but not by subcutaneous injection of 10 mg of octylphenol (Fig 4.1, 4.2). The decrease in thymocyte numbers seen in the C57BL/6 mice injected with octylphenol was also significantly different from the thymocyte numbers of mice fed octylphenol (Fig 4.1). Similarly, the decrease in the % body weight of the thymus in mice injected with 17 $\beta$ -estradiol was also significantly different from the percent body weight of the thymus of mice fed 17 $\beta$ -estradiol in the C57BL/6 males (Fig 4.2). In contrast, the decrease in the thymocyte cellularity seen in both the B/W and C57BL/6 males injected with 17 $\beta$ -estradiol and the decrease in the percent body weight of the thymus in the B/W males injected with 17 $\beta$ -estradiol was not significantly different from the thymocyte cellularity and % body weight of the thymus in mice fed 17 $\beta$ -estradiol (Fig 4.1, 4.2).

Although the injection of 10 mg of octylphenol and 17 $\beta$ -estradiol resulted in decreased thymic cellularity, no significant changes in the proportion of immature (CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup>) and mature (CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup>) thymocytes were noted in the C57BL/6 males (Fig 4.3). In contrast, injection of 17 $\beta$ -estradiol in the B/W males resulted in an increase in the presumably mature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes compared to control mice, but not compared to mice fed 17 $\beta$ -estradiol (Fig 4.3). The injection of 10 mg of octylphenol in B/W males did not result in any change in the proportion of immature or mature thymocytes (Fig 4.3).

**Effect of octylphenol on apoptosis of thymocytes:** The level of apoptosis of freshly isolated thymocytes and of cultured thymocytes stimulated with anti-CD3 antibodies and PMA+I was determined. The data were analyzed and are presented as the change from the media baseline of the thymocytes stimulated with the anti-CD3 or PMA+I (Anti-CD3 or PMA+I – Media). The level of apoptosis was assessed by the proportion of live (7-AAD<sup>dull</sup>), early apoptotic (7-AAD<sup>intermediate</sup>), late apoptotic/necrotic (7-AAD<sup>bright</sup>), and total dying (7-

AAD<sup>intermediate+bright</sup>) cells. The feeding or injection of octylphenol or 17 $\beta$ -estradiol did not affect the level of apoptosis in freshly isolated thymocytes in either the C57BL/6 or B/W males (Fig 4.4). The proportion of live or apoptotic thymocytes at 4, 12, or 24 hours of culture with anti-CD3 or PMA+I was not affected by the feeding or injection of octylphenol or the feeding of 17 $\beta$ -estradiol in either the C57BL/6 or B/W males (Fig 4.5-4.12). The *in vivo* injection of 17 $\beta$ -estradiol did effect the change from media in the level of apoptosis of both strains at 24 hours of culture. Thymocyte isolated from C57BL/6 male injected with 17 $\beta$ -estradiol and stimulated with anti-CD3 had a significantly greater increase from media in the proportion of live cells (7-AAD<sup>dull</sup>) than the controls (Fig 4.7). Thymocytes isolated from B/W males injected with 17 $\beta$ -estradiol and stimulated with PMA+I had a significantly lower increase from media in the proportion of late apoptotic/necrotic cells (7-AAD<sup>bright</sup>) (Fig 4.10).

**Effect of octylphenol on bone marrow cellularity:** Neither the feeding nor the injection of 10 mg of octylphenol caused a change in the bone marrow cellularity in either the C57BL/6 or B/W males (Fig 4.13). In contrast, the injection of 17 $\beta$ -estradiol caused a significant decrease in bone marrow cellularity compared to controls in both strains (Fig 4.13). However, the bone marrow cellularity was not significantly different between mice fed 17 $\beta$ -estradiol and mice injected with 17 $\beta$ -estradiol (Fig 4.13). Bone marrow was also double stained for GR1 and CD11b/F480 and analyzed by flow cytometry to determine the proportion of developing granulocytes, monocytes, and macrophages in the bone marrow. The data are represented as a dot plot because n=2 for each treatment. The injection of 10 mg of octylphenol resulted in an increase in the GR1+CD11b/F480<sup>+</sup> cells in C57BL/6 males (Fig 4.14) and an increase in the GR1+CD11b/F480<sup>+</sup> and the GR1-CD11b/F480<sup>+</sup> in the B/W males (Fig 4.14).

**Effect of octylphenol on dsDNA autoantibody production:** The level of anti-dsDNA autoantibodies in the serum was not affected by the feeding or the injection of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol in the C57BL/6 or B/W mice (Fig 4.15). Both the C57BL/6 and B/W mice exhibited detected levels of IgM isotype anti-dsDNA antibodies; however, the average absorbance for the B/W males was higher than the average absorbance for the C57BL/6 males, and the levels detected in the treatment groups did not differ from the controls in either strain (Fig 4.15). The level of IgG isotype anti-dsDNA antibodies in the serum, also, did not differ between the control group and the treatment groups in either strain (Fig 4.15). Furthermore, in both the C57BL/6 and B/W mice neither the level of the IgG nor the IgM anti-dsDNA antibodies in the serum

differed between the mice fed octylphenol or 17 $\beta$ -estradiol and those injected with octylphenol or 17 $\beta$ -estradiol, respectively (Fig 4.15).

**Effect of octylphenol on non-lymphoid tissue:** The liver, kidneys, salivary glands, heart, and lungs from all mice were examined histopathologically. One out of 6 of the B/W mice in the injected control group was diagnosed with purulent sialoadenitis because of an inflammation of the salivary glands that could have been caused by a bacterial infection. One out of 5 C57BL/6 mouse fed octylphenol had hydronephrosis and pyelonephritis in the kidney that is indicative of a chronic infection of the lower urinary tract. The kidneys, salivary glands, heart, and lungs from all other mice were found to be normal.

Injection of 10 mg of octylphenol resulted in hepatic hematopoiesis in both the C57BL/6 and B/W males (Table 4.1). Six out of the six livers from C57BL/6 mice and 5 out of 5 livers from B/W mice injected with octylphenol showed hematopoiesis upon histopathological examination (Table 4.1). One out of the seven C57BL/6 mice fed 17 $\beta$ -estradiol and 1 out of the 5 fed octylphenol also exhibited minimal hematopoiesis of the liver (Table 4.1). No treatment other than the injection of octylphenol resulted in signs of hepatic hematopoiesis in the B/W mice (Table 4.1).

The injection of 10 mg of octylphenol also resulted in severe inflammation and necrosis of the skin in both strains. Raised areas, severe adhesions and scabbing were noted at the sites of injection in all mice injected with octylphenol, but not in mice injected with the oil/ethanol control or 17 $\beta$ -estradiol. Sections of the skin from mice injected with octylphenol and control mice were examined histopathologically. The skin sections from control mice injected with oil/ethanol were found to be normal. The skin sections from B/W and C57BL/6 mice injected with octylphenol were diagnosed with necrotizing, suppurative panniculitis with fibrosis and dermal necrosis, which could have been caused by bacterial infections. Corn oil smeared on blood agar plates ensure sterility. One B/W mouse lost the function of its hind leg during the third week of treatment. Muscle sections from the leg sent for histopathological examination also showed necrosis with surrounding inflammation and infiltration of lymphocytes and neutrophils. Final treatments with octylphenol were injected on the lower back close to the hind limbs because of adhesions and scabs on the upper back. Long-term studies utilizing subcutaneous injection of 10 mg of octylphenol were thus abandoned.

**Effect of octylphenol on the morphology of the spleen:** Subcutaneous injection of 10 mg of octylphenol resulted in a significant increase compared to controls and to mice fed octylphenol in both the size and cellularity of the spleen in both the C57BL/6 and B/W males (Fig 4.16, 4.17). The percent body weight of the spleen was increased more than 3-fold in both strains (Fig 4.16) and the cellularity following lysis of the red blood cells was increased more than 4-fold in both strains (Fig 4.17). The injection of  $17\beta$ -estradiol did not result in any change in the percent body weight of the spleen or in the cell numbers following lysis of the red blood cells (Fig 4.16, 4.17).

Subcutaneous injection of octylphenol also resulted in significant changes in the proportion of T cell and B cell markers in the splenic cells from both the C57BL/6 and B/W males (Fig 4.18). The proportion of both the Thy1.2<sup>+</sup> and the  $\alpha\beta$ -TCR<sup>+</sup> T cells was significantly reduced by more than half in mice subcutaneously injected with octylphenol compared to controls in both strains (Fig 4.18). Furthermore, the proportion of Thy1.2<sup>+</sup> T cells in the B/W mice injected with octylphenol and the proportion of  $\alpha\beta$ -TCR<sup>+</sup> T cells in the B/W and C57BL/6 mice injected with octylphenol, were significantly lower than those seen in mice fed octylphenol (Fig 4.18). The proportion of CD19<sup>+</sup> B cells was also decreased significantly compared to control mice and mice fed octylphenol in the C57BL/6 model, but only compared to control mice in the B/W model (Fig 4.18). A comparable, significant increase in the proportion of Thy1.2<sup>-</sup>CD19<sup>-</sup> cells was seen in mice from both strains injected with octylphenol compared to controls and mice fed octylphenol (B/W: control –  $14.2 \pm 1.2$ , 10 mg octylphenol injected –  $53.0 \pm 3.4$ , 10 mg octylphenol fed –  $17.5 \pm 3.5$ ; C57BL/6: control –  $15.0 \pm 2.9$ , 10 mg octylphenol injected –  $54.5 \pm 2.2$ , 10 mg octylphenol fed –  $10.8 \pm 1.5$ ). The injection of  $17\beta$ -estradiol did not result in any change in the proportion of Thy1.2,  $\alpha\beta$ -TCR, or CD19 positive cells in either strain (Fig 4.18). However, injection of  $17\beta$ -estradiol did cause a significant increase in the proportion of Thy1.2<sup>-</sup>CD19<sup>-</sup> cells compared to controls and compared to those mice fed  $17\beta$ -estradiol in the B/W males (control –  $14.2 \pm 1.2$ ;  $17\beta$ -estradiol injected –  $27.9 \pm 2.7$ ,  $17\beta$ -estradiol fed –  $16.0 \pm 3.2$ ).

A few spleens were also examined histopathologically because of the drastic increase in the percent body weight and cellularity of the spleen and decrease in T cell and B cell markers. Excessive hematopoiesis was noted in each spleen from C57BL/6 and B/W males injected with 10 mg of octylphenol (n=4) and spleens from all other treatments were normal. One spleen from a C57BL/6 mouse injected with octylphenol was diagnosed with granulocytic leukemia.



Because preliminary results suggested that the increase in splenic cellularity was due to excessive hematopoiesis with an increase in granulocyte precursors, the proportion of granulocytes in the spleen was assessed by flow cytometric analysis of cell markers in the final experiments for both the C57BL/6 and B/W males. CD71 a developmental marker for granulocytes, monocytes, and macrophages, but it is also expressed on activated T cells, B cells, and NK cells. Therefore, spleen cells were double stained with PE-anti-CD71 antibodies and FITC-anti-Thy1.2 plus FITC-anti-CD45Rb antibodies to rule out the activated T and B cells expressing CD71 and assess the proportion of developing granulocytes, monocytes, and macrophages in the spleen. GR1 and CD11b are also developmental markers expressed on granulocytes, monocytes, and macrophages, and F480 is expressed on monocytes. Spleen cells were double stained with PE-anti-GR1 antibodies and FITC-anti-F480 antibodies plus FITC-anti-CD11b antibodies to further assess the proportion of developing granulocytes, monocytes and macrophages in the spleen. The data are reported as dot plots due to the small sample size (n=2). Injection with 10 mg of octylphenol seemed to result in an increase in the proportion of CD71 single positive cells (CD71<sup>+</sup>Thy1.2/CD45Rb<sup>-</sup>) in both the C57BL/6 and B/W mice, but not in the proportion of CD71 cells double stained with the T cell and B cell markers (CD71<sup>+</sup>Thy1.2/CD45Rb<sup>+</sup>) (Fig 4.19). Injection of octylphenol in both strains of mice also seemed to result in an increase in the proportion of GR1<sup>+</sup> cells that double stained with CD11b and F480 (GR1<sup>+</sup>CD11b/F480<sup>+</sup>) (Fig 4.20). No other treatment affected the proportion of staining of CD71, GR1, CD11b, or F480 (Fig 4.19, 4.20).

The expression of CD69 was also examined in the splenic cells after 4 hours of stimulation with PMA+I. The expression of CD69 was significantly decreased compared to controls and mice fed octylphenol in both the C57BL/6 and B/W males that were subcutaneously injected with octylphenol (Fig 4.21). The injection of 17 $\beta$ -estradiol also significantly decreased the expression of CD69 compared to control, but not compared to the feeding of 17 $\beta$ -estradiol in B/W males (Fig 4.21). To assess the relationship between an increase in the number of developing granulocytes in the spleen and the decreased expression of CD69 upon stimulation, splenic cells which were stimulated for 4 hours with PMA+I, were double stained with FITC-anti-CD69 antibodies and PE-anti-GR1 antibodies in the final B/W and C57BL/6 experiments. Injection with octylphenol seemed to result in a decrease in the proportion of CD69<sup>+</sup>GR1<sup>-</sup> cells and an increase in the proportion of CD69<sup>-</sup>GR1<sup>-</sup> and CD69<sup>-</sup>GR1<sup>+</sup> cells in both the C57BL/6 and B/W males (Fig 4.22).

**Effect of octylphenol on reproductive tissue:** Reproductive changes were seen in C57BL/6 male subcutaneously injected with 10 mg of octylphenol, but not in B/W males injected with octylphenol. Neither strain had changes in the percent body weight of the testis (Fig 4.23); however, C57BL/6 mice injected with octylphenol had a significant decrease in percent body weight of the seminal vesicles compared to controls and mice fed octylphenol, whereas the B/W males did not (Fig 4.24). Subcutaneous injection of  $17\beta$ -estradiol, also, did not result in significant changes in the percent body weight of the testis in either strain (Fig 4.23). However, subcutaneous injection of  $17\beta$ -estradiol did result in a significant decrease in the percent body weight of the seminal vesicles compared to controls in both the C57BL/6 and B/W males (Fig 4.24). Furthermore, the percent body weight of the seminal vesicle in the B/W mice injected with  $17\beta$ -estradiol was significantly different from that of B/W mice fed  $17\beta$ -estradiol; whereas the percent body weight of the seminal vesicle does not differ between the C57BL/6 fed  $17\beta$ -estradiol and those injected with  $17\beta$ -estradiol (Fig 4.24). The testis and seminal vesicles from all treatment groups were found to be normal by histopathological examination.

#### **e.) Discussion**

The injection of 10 mg of octylphenol led to changes in the spleen, thymus, and reproductive tract; however, the results were complicated by the inflammation and necrosis that developed around the sites of injection. Every C57BL/6 and B/W mouse that was injected with octylphenol developed skin lesions and skin lesions were not found on mice from any other treatment group. The lesions were diagnosed with necrotizing, suppurative panniculitis with fibrosis and dermal necrosis, and may have been caused by bacterial infections. The oil, which was utilized for injections in all treatment groups, was analyzed and found to be sterile. Octylphenol is corrosive chemical and could have initiated necrosis of the tissue upon injection allowing for bacterial infection to take hold. The presence of such severe skin lesions may affect immune parameters because of the stress induced, or if a bacterial infection is present immune parameters will be affected because of changes induced in the immune system to fight the infection. Therefore, any changes in the immune parameters tested could be due to the estrogenic or non-estrogenic properties of octylphenol, to the infection or stress, or to a combination of the properties of octylphenol and the stress and infection from the skin lesions and interactions between the two.

Injection of 10 mg of octylphenol led to a decrease in thymic cellularity and a decrease in seminal vesicle weights in the C57BL/6 mice, but not in the B/W mice. Since both strains had skin lesions and the thymic changes were only

seen in the C57BL/6 mice, it might suggest that these changes are due to the estrogenic properties of octylphenol. However, it is also possible that the B/W mice are less vulnerable to the effects of stress, which is known to cause thymic atrophy, or are less vulnerable to infections. Furthermore, although no changes were noted in the thymus or reproductive tract of the B/W males, it could be argued that the skin lesions altered the metabolism of octylphenol. Therefore, definitive conclusions can not be drawn about the effects of the injection of 10 mg of octylphenol on the thymus or reproductive tract in our model.

Both the C57BL/6 and B/W males that were injected with 10 mg of octylphenol developed abnormal hematopoiesis in the liver and spleen. The hematopoiesis seen could have been induced to aid the bone marrow in the production of granulocyte, monocyte, and macrophage precursors as a result of the severe inflammation of the skin. However, estrogen treatment has also been shown to increase mononuclear cells in the liver (Okayama et al, 1992). The mononuclear cells include  $\alpha\beta$ T cells with intermediate TCR, especially forbidden autoreactive T cell oligoclonal and  $\nabla\beta 8^+$  cells characterized as extrathymic  $\alpha\beta$ T cells (Okayama et al, 1992). The increase in granulocyte precursors that was noted in histopathological examination of the spleen agrees with cell surface marker data from flow cytometric analysis. The decrease in the proportion of T cells and B cells is likely due to the increase in the CD71<sup>+</sup> and GR1<sup>+</sup>CD11b/F480<sup>+</sup> developing granulocytes, monocytes, and macrophages in the spleen. Furthermore, the decrease in the expression of CD69 on stimulated spleen cells could be due to the decrease in T cells and B cells, which express CD69 upon stimulation, and an increase in a population of cells, such as the GR1<sup>+</sup> developing granulocytes, monocytes, and macrophages, that may not express CD69 upon stimulation with PMA+I. An increase in developing granulocytes, monocytes, and macrophages (GR1<sup>+</sup>CD11b/F480<sup>+</sup>) was also seen in the bone marrow of both strains, along with an increase in the CD11b/F480 single positive cells in the B/W mice. It is possible that these changes are due to severe inflammation or infection in the skin, which may stimulate the overproduction of granulocytes and monocytes. However, in the B/W mice the decreased expression of CD19 and in the C57BL/6 mice the decreased expression of Thy1.2, which were significantly different from the control mice, were not significantly different from mice fed octylphenol. Therefore, the levels of CD19 and Thy1.2 expression on lymphocytes from mice fed octylphenol must have been decreased enough that they were not significantly different from the lymphocytes from mice injected with octylphenol. This suggests a mechanism related to the properties of octylphenol, because the mice fed octylphenol did not have skin

lesions. Therefore, definite conclusions can not be drawn about cause of the changes seen in the spleens of mice injected with 10 mg of octylphenol.

The injection of  $17\beta$ -estradiol induced the expected atrophy of the thymus in both the C57BL/6 and B/W mice; however, a decrease in the immature cell ( $CD4^+CD8^+$ ), which has been shown by many to be the cause of the atrophy seen in estrogenic treatment, was not noted. It has been suggested that the decrease in the number of immature T cells in the thymus is due to a decrease in bone marrow cellularity upon estrogen treatment, which leads to a decrease in the migration of precursors to the thymus. A decrease in the bone marrow cellularity was noted in both strains upon the injection of  $17\beta$ -estradiol, along with an increase in the  $CD8^+$  mature thymocytes in the B/W mice. Changes in CD69 expression and apoptosis of cultured thymocytes, neither of which have been previously reported with estrogen treatment, were also noted in our study. In the B/W mice, there was a decrease in the expression of CD69 upon stimulation of the spleen cells isolated with the injection of  $17\beta$ -estradiol. This is in agreement with unpublished data in our lab in which a decrease in CD69 expression on stimulated spleen cells is noted with long-term estrogen treatment by subcutaneous implant. Furthermore, in the kinetic studies of apoptosis of thymocytes, at 24 hours of culture there was a decrease in the population of late apoptotic/necrotic cells in thymocytes from the B/W mice injected with  $17\beta$ -estradiol and an increase in the population of live cells in thymocytes isolated from C57BL/6 mice injected with  $17\beta$ -estradiol. This may indicate that with estrogen treatment there are fewer cells dying at 24 hours of culture because most have already died and the apoptotic rate has decreased compared to controls or the population of cells normally dying at this time point are rescued from apoptosis. However, no changes in the proportions of live and apoptotic cells were noted with estrogen treatment at any earlier time points, and no significant changes were noted in the other populations of cells at the 24 hour time point. Therefore, these may be incidental findings that have no bearing on the functioning of the apoptotic signaling in thymocytes from mice injected with  $17\beta$ -estradiol. The injection of  $17\beta$ -estradiol did not result in histopathological changes in the kidney, liver, or salivary glands or in increased levels of anti-dsDNA autoantibodies in the serum to indicate an accelerated onset of autoimmune disease. As discussed in Chapter 3, the mice may have been too young to have pathological evidence of accelerated autoimmune changes, especially because they are not orchietomized.

The second aim of this study was to determine whether or not feeding and injection of octylphenol or  $17\beta$ -estradiol have differential effects on the immune

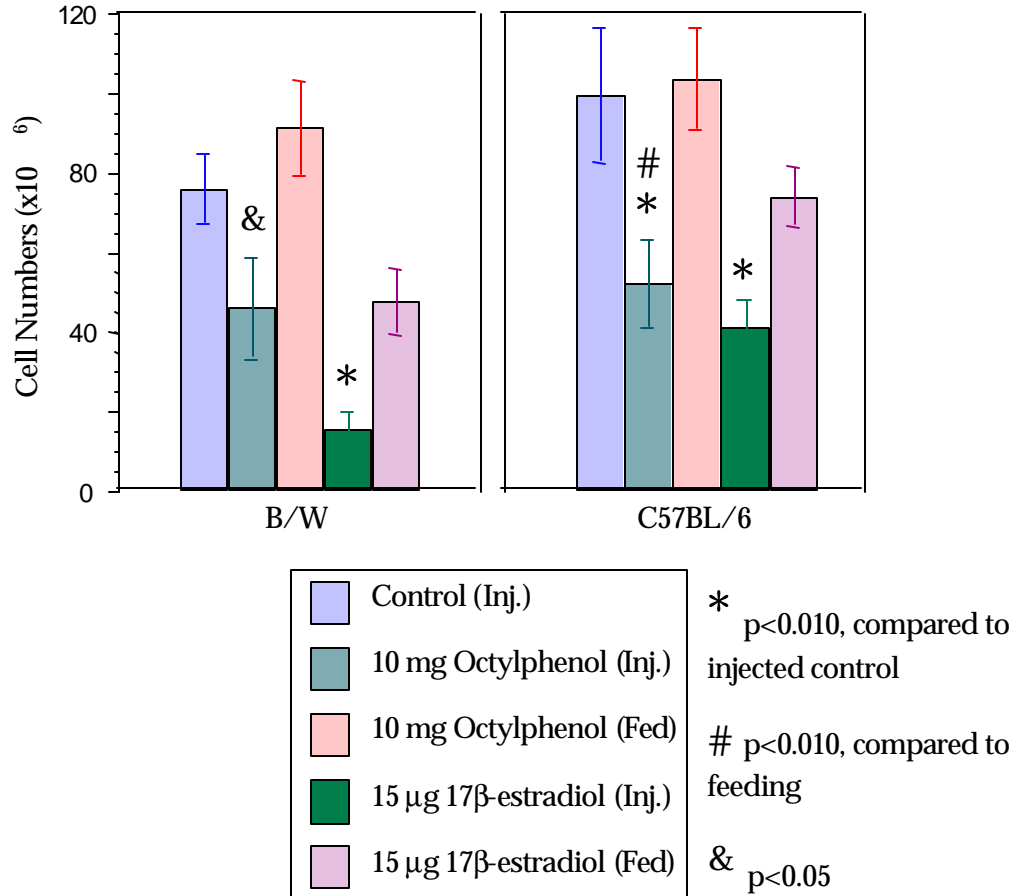
parameters studied. Due to the complications involved in the mice injected with octylphenol, definitive conclusions can not be drawn about whether or not feeding of octylphenol has the same effects on the thymus and reproductive tract. However, one can still compare the feeding and injection of 17 $\beta$ -estradiol. Of all the changes noted from the injection of 17 $\beta$ -estradiol that were significantly different from control mice, only three were also significantly different from mice fed 17 $\beta$ -estradiol. The percent body weight of the thymus in C57BL/6 mice, the percent body weight of the seminal vesicles in the B/W mice, and the proportion of Thy1.2-CD19<sup>-</sup> spleen cells in the B/W mice were significantly different between the mice fed 17 $\beta$ -estradiol and those injected with 17 $\beta$ -estradiol. All other changes were not significantly different between the routes of administration. As can be seen from the graphs of these data, the feeding of 17 $\beta$ -estradiol results in data that lies between the data from control mice and the data from 17 $\beta$ -estradiol injected mice. This suggests that these changes seen in the thymus and reproductive tract from feeding 17 $\beta$ -estradiol are similar to the changes seen with the injection of 17 $\beta$ -estradiol, but are of a lesser extent because of different routes of administration.

Furthermore, the significant difference in percent body weight of the thymus in the C57BL/6 mice and the percent body weight of the seminal vesicles in the B/W mice between mice that were fed and injected with 17 $\beta$ -estradiol, may suggest organs with differing sensitivity to the estrogenic effects of oral 17 $\beta$ -estradiol in each strain. The significant difference in results between the feeding and injection of 17 $\beta$ -estradiol indicates that the mice fed 17 $\beta$ -estradiol did not have changes similar to the mice injected with 17 $\beta$ -estradiol. In contrast, when a significant difference is seen between the control mice and mice injected with 17 $\beta$ -estradiol, but a significant difference is not seen between the mice fed 17 $\beta$ -estradiol and those injected with 17 $\beta$ -estradiol, it indicates similar changes in the fed and injected mice. Therefore, the feeding of 17 $\beta$ -estradiol did not result in the estrogenic decrease in the percent body weight of the thymus similar to the injected mice in the C57BL/6 model, but it did in the B/W model. In contrast, the feeding of 17 $\beta$ -estradiol did not result in the estrogenic decrease in the % body weight of the seminal vesicles seen in the injected mice in the B/W model, but did in the C57BL/6 model. This suggests that the thymus in the B/W males may be more sensitive to oral estrogenic treatment than the reproductive tract, and the reproductive tract in the C57BL/6 males may be more sensitive to oral estrogenic treatment than the thymus.

Future studies could attempt to decipher whether the changes noted in the spleen were the result of octylphenol treatment or the result of skin lesions

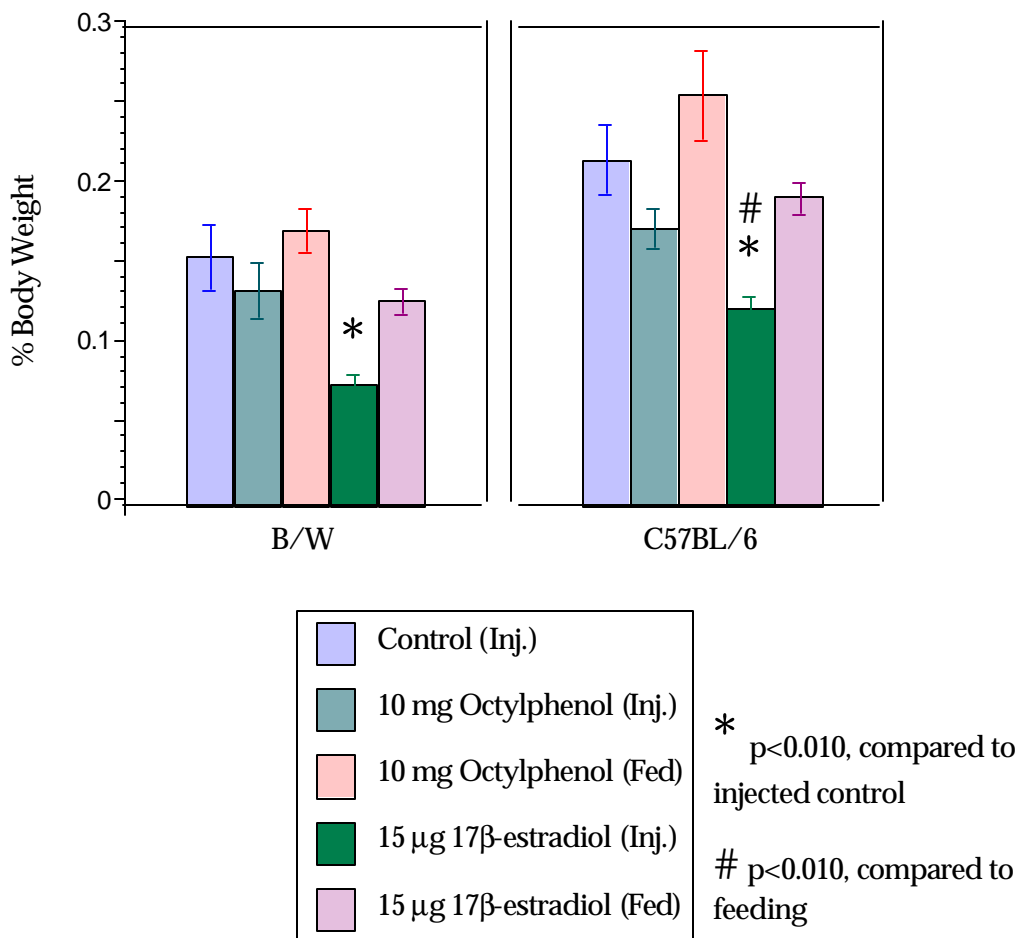
caused by the subcutaneous injection of octylphenol. It would be necessary to find a dose of octylphenol that was able to induce the changes in the spleen without causing the skin lesions. However, subcutaneous injection is not a relevant route of exposure to octylphenol, therefore it is questionable whether it is necessary to determine if the splenic changes are due to the injection of octylphenol. These studies were conducted to determine whether octylphenol is estrogenic in our model as compared to other models tested, because we did not find any estrogenic effects of octylphenol in previous studies utilizing oral dosing. We found that we are unable to study the effects of 10 mg of octylphenol subcutaneously injected in our model because of skin lesions that develop. Therefore, future studies may want to focus on another model, such as another age group or females instead of males, or another relevant route of exposure, such as dermal exposure.

### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Cellularity of the Thymus in B/W and C57BL/6 Mice



**Fig. 4.1:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment both strains had the thymus removed. The thymocytes were isolated and counted.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected; n=6 for all other groups)

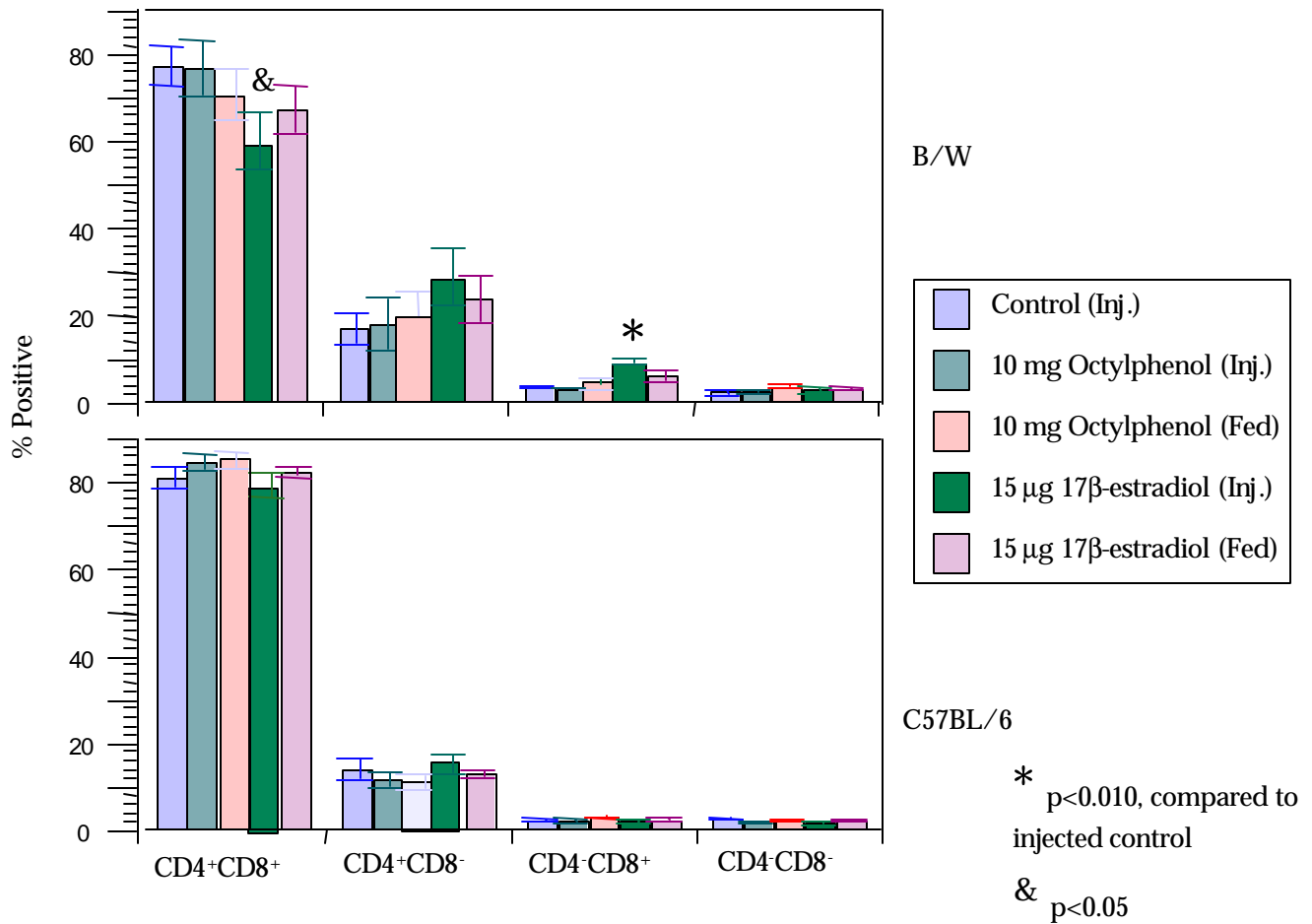
### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Thymus in B/W and C57BL/6 Mice



**Fig. 4.2:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment both strains were weighed and the thymus was removed. The thymus was weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6:  $n=5$  for 10 mg octylphenol fed;  $n=6$  for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed;  $n=7$  for 17 $\beta$ -estradiol fed; B/W:  $n=5$  for 10 mg octylphenol injected;  $n=6$  for all other groups)

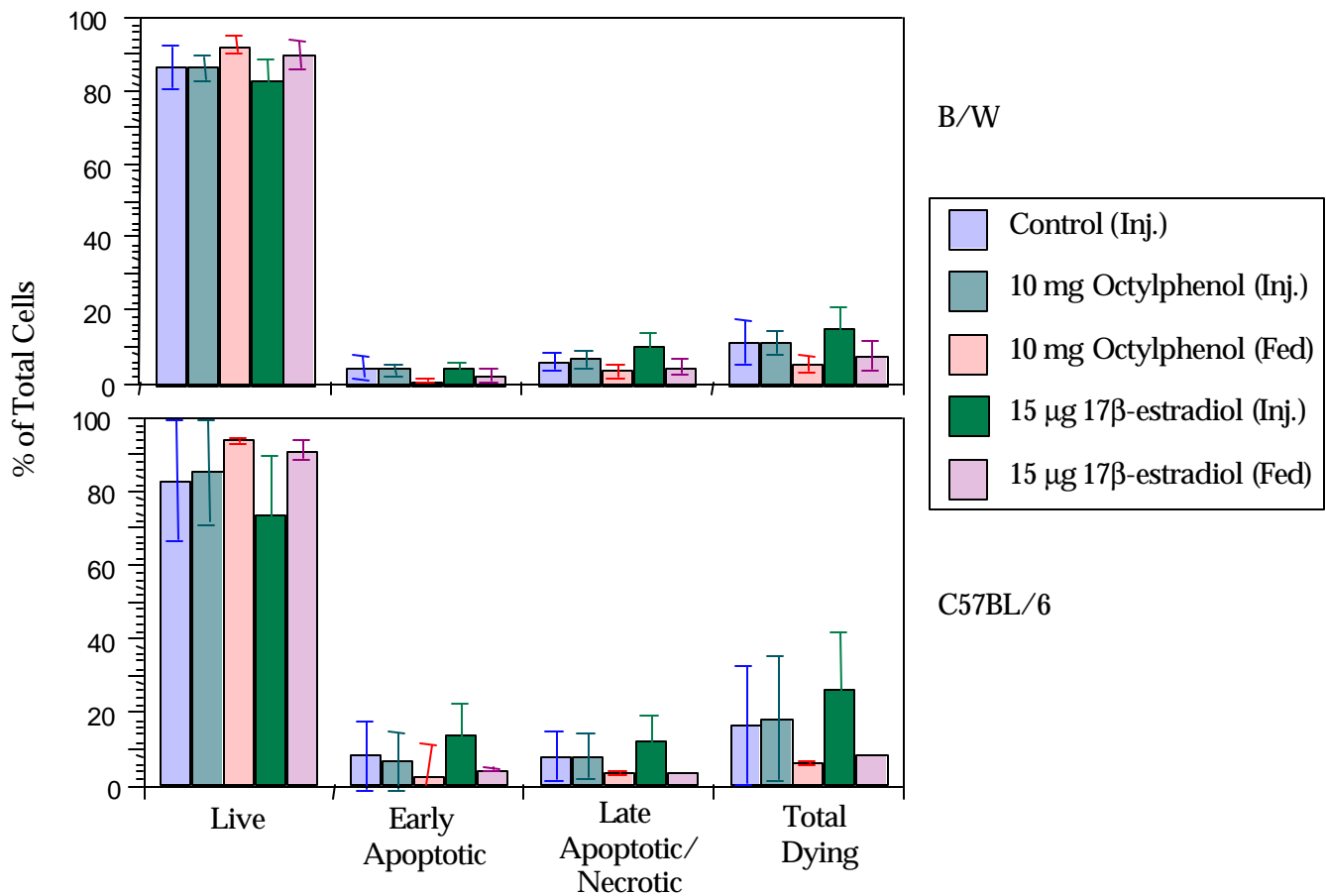


### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Proportion of Mature and Immature Thymocytes in B/W and C57BL/6 Mice



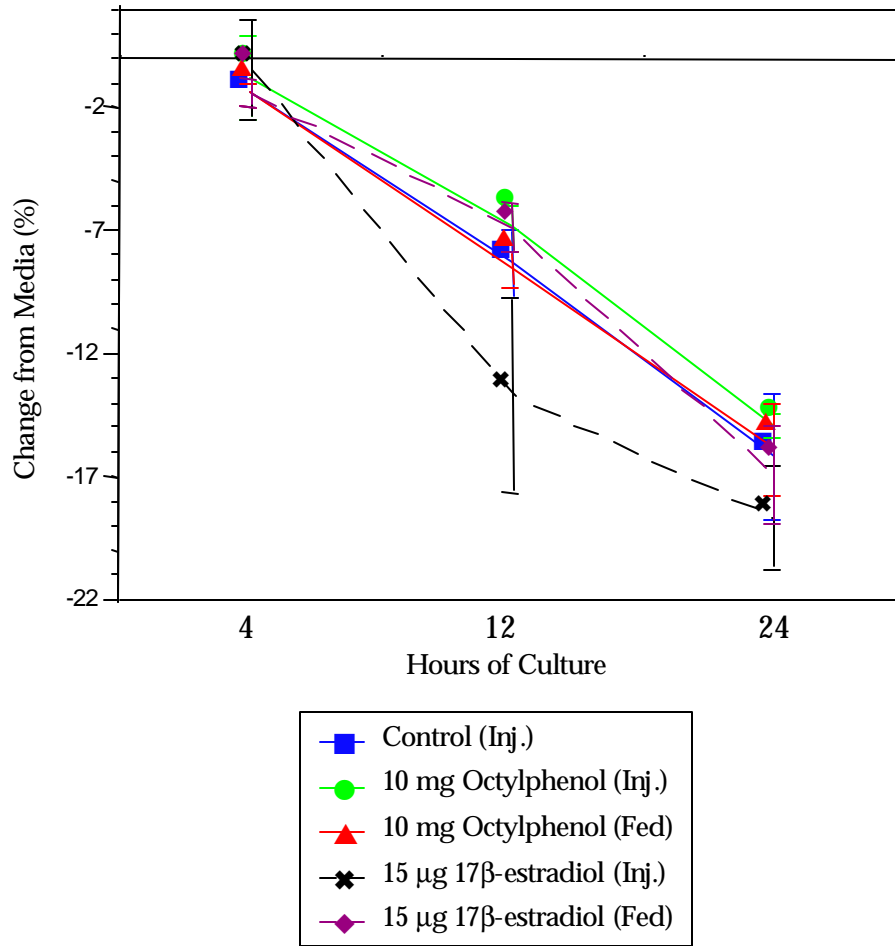
**Fig. 4.3:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymocytes were isolated from both strains and stained with PE-conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8a antibodies or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected; n=6 for all other groups)

### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on Apoptosis of Freshly Isolated Thymocytes in B/W and C57BL/6 Mice



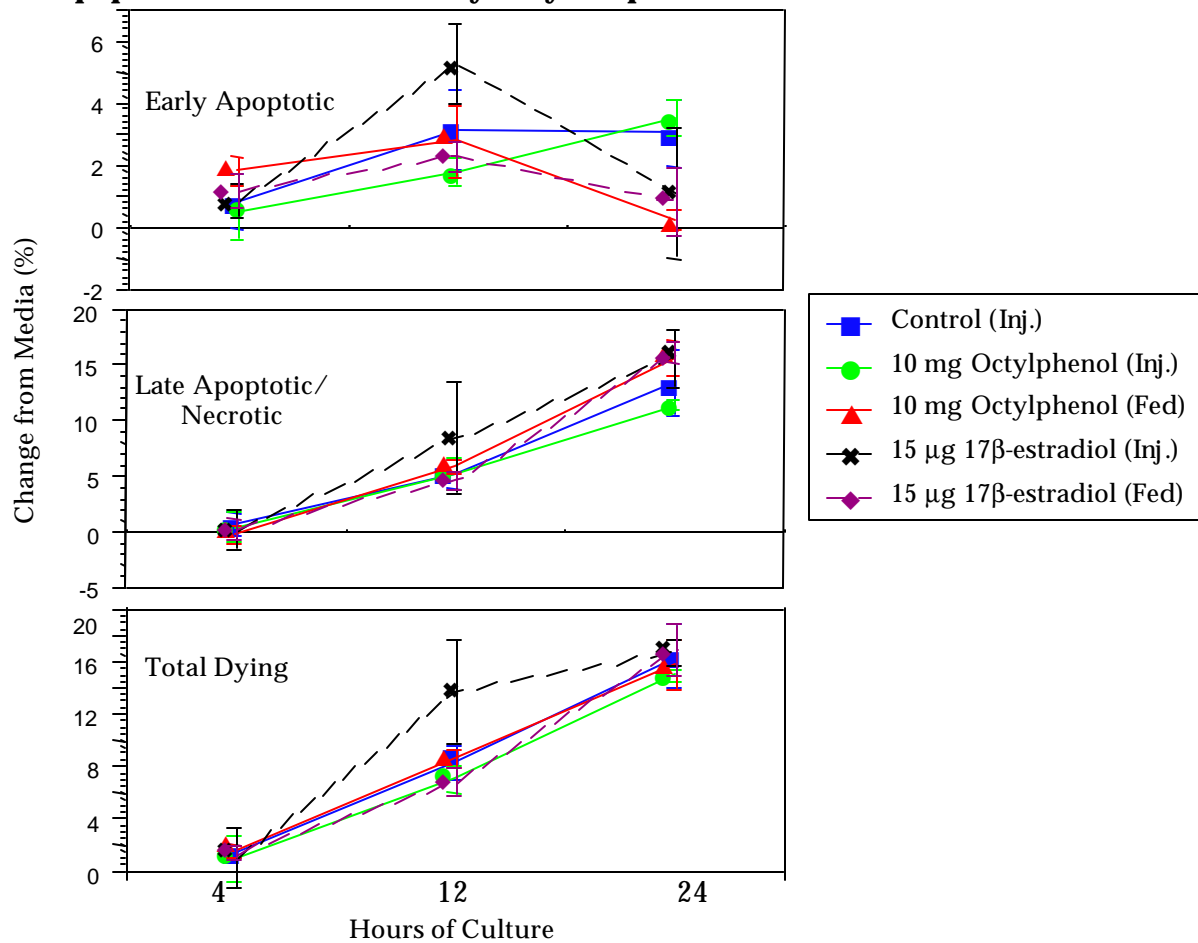
**Fig. 4.4:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are presented as the percentage of cells that are 7-AAD<sup>dull</sup> (live), 7-AAD<sup>intermediate</sup> (early apoptotic), and 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying).  $p \leq 0.010$  was considered significant. (C57BL/6:  $n=3$  for 10 mg octylphenol injected,  $n=2$  for all other groups; B/W:  $n=3$  for octylphenol injected;  $n=4$  for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in B/W Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon Anti-CD3 Stimulation**



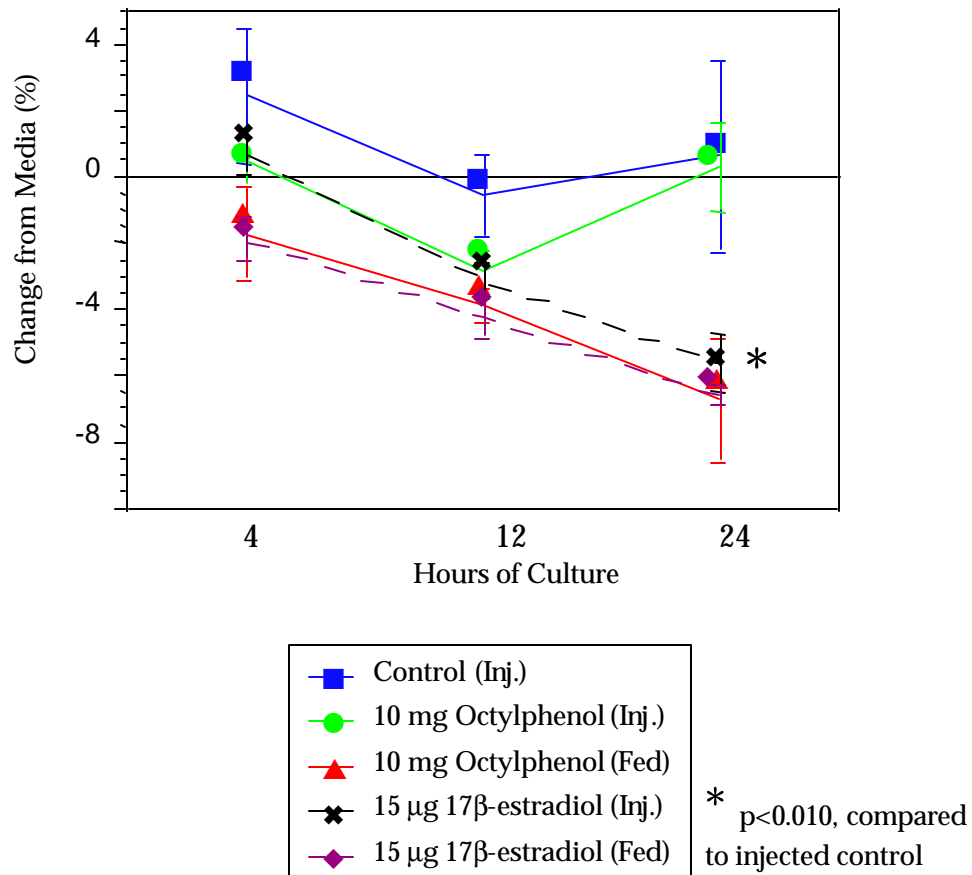
**Fig. 4.5:** Eight to ten-week old B/W males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for 10 mg octylphenol injected at 4, 12, and 24 hours and 17 $\beta$ -estradiol injected at 4 and 12 hours; n=4 for all other groups and time points)

### Effect of Octylphenol Treatment in B/W Mice on the Apoptosis and Necrosis of Thymocytes upon Anti-CD3 Stimulation



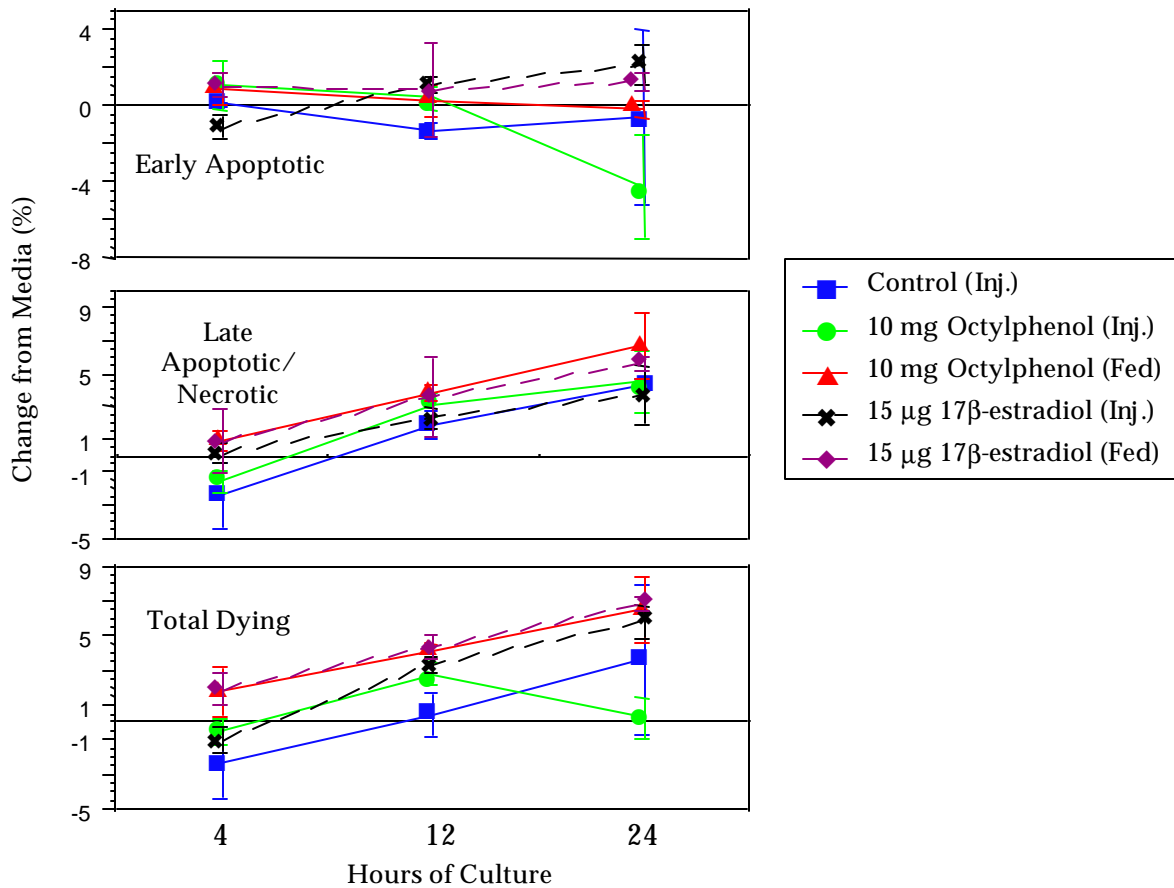
**Fig. 4.6:** Eight to ten-week old B/W males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15 µg 17β-estradiol, oral dosing of 10 mg of octylphenol or 15 µg of 17β-estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the anti-CD3 stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. ( $n=3$  for 10 mg octylphenol injected at 4, 12, and 24 hours and 17β-estradiol injected at 4 and 12 hours;  $n=4$  for all other groups and time points)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in C57BL/6 Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon Anti-CD3 Stimulation**



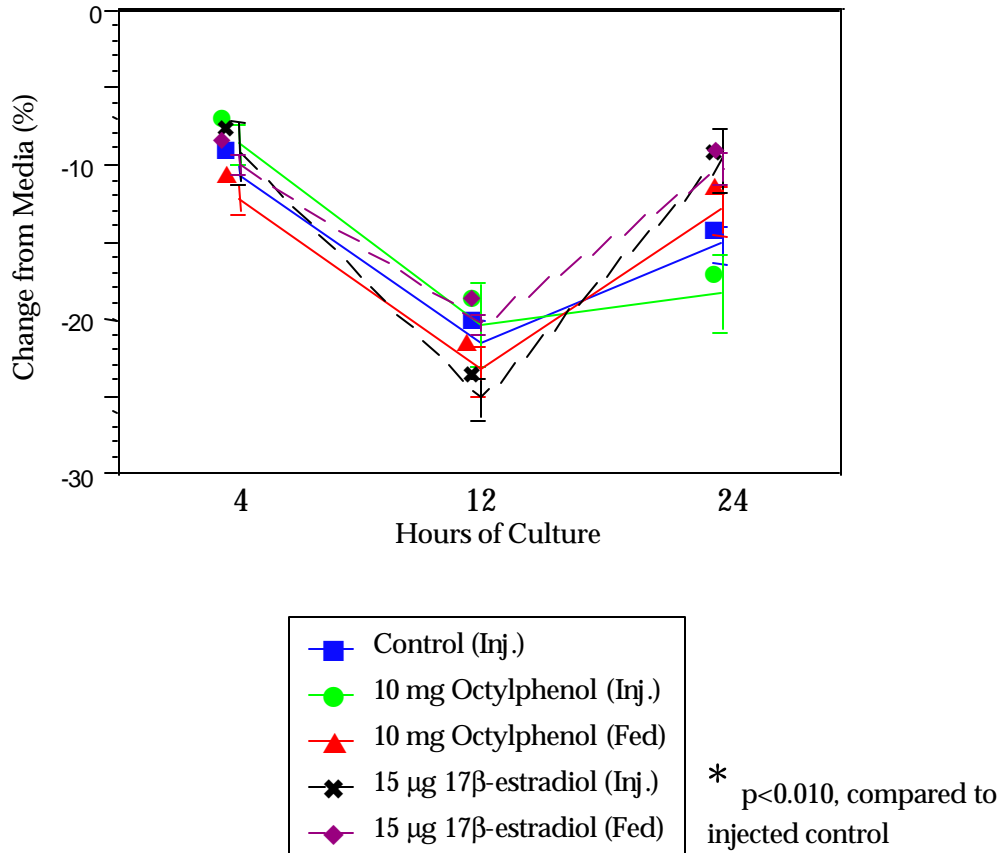
**Fig. 4.7:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for control, n=4 for 10 mg octylphenol injected, 10 mg octylphenol fed, and 17 $\beta$ -estradiol fed; n=5 for 17 $\beta$ -estradiol injected)

**Effect of Octylphenol Treatment in C57BL/6 Mice on the Apoptosis and Necrosis of Thymocytes upon Anti-CD3 Stimulation**



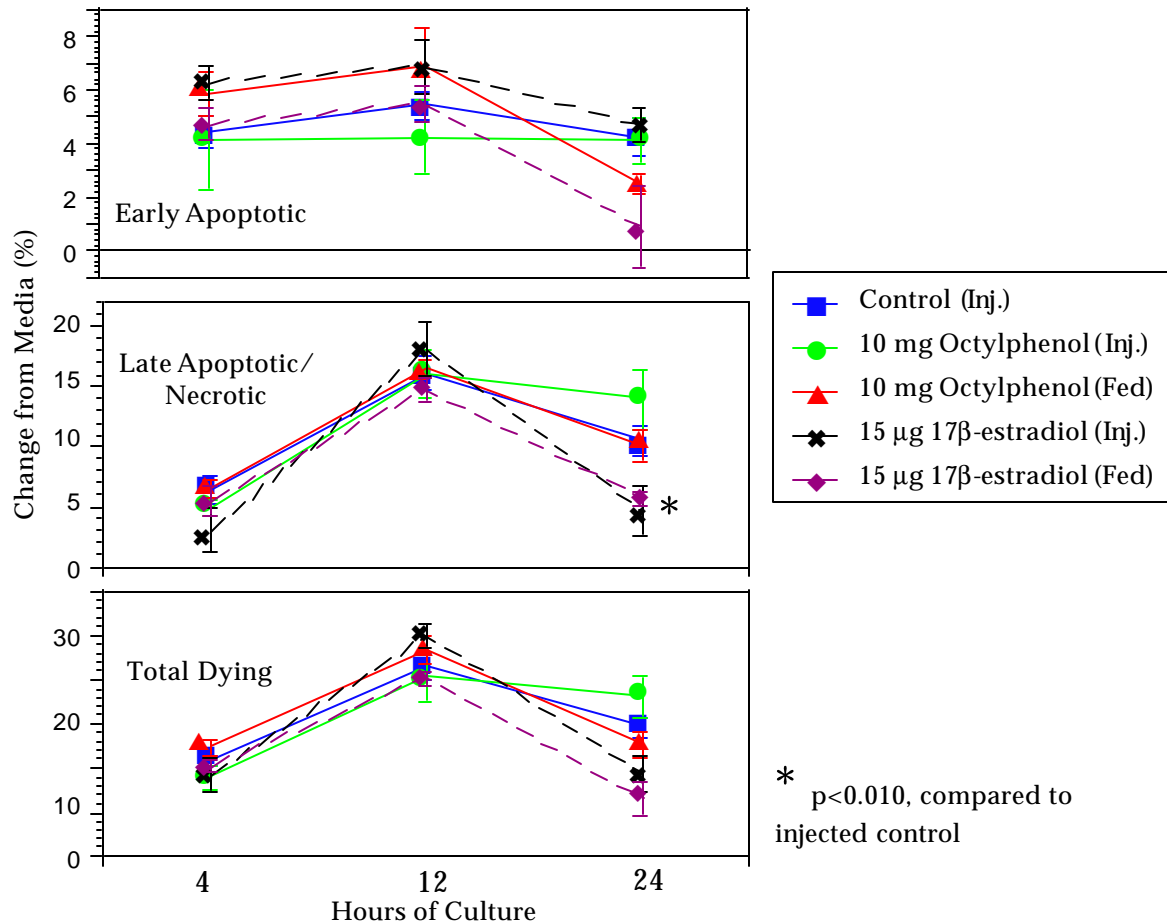
**Fig 4.8:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15 µg 17β-estradiol, oral dosing of 10 mg of octylphenol or 15 µg of 17β-estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with anti-CD3 antibodies or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the anti-CD3 stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for control, n=4 for 10 mg octylphenol injected, 10 mg octylphenol fed, and 17β-estradiol fed; n=5 for 17β-estradiol injected)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in B/W Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon PMA+I Stimulation**



**Fig. 4.9:** Eight to ten-week old B/W males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for 10 mg octylphenol injected at 4, 12, and 24 hours and 17 $\beta$ -estradiol injected at 12 hours; n=4 for all other groups and time points)

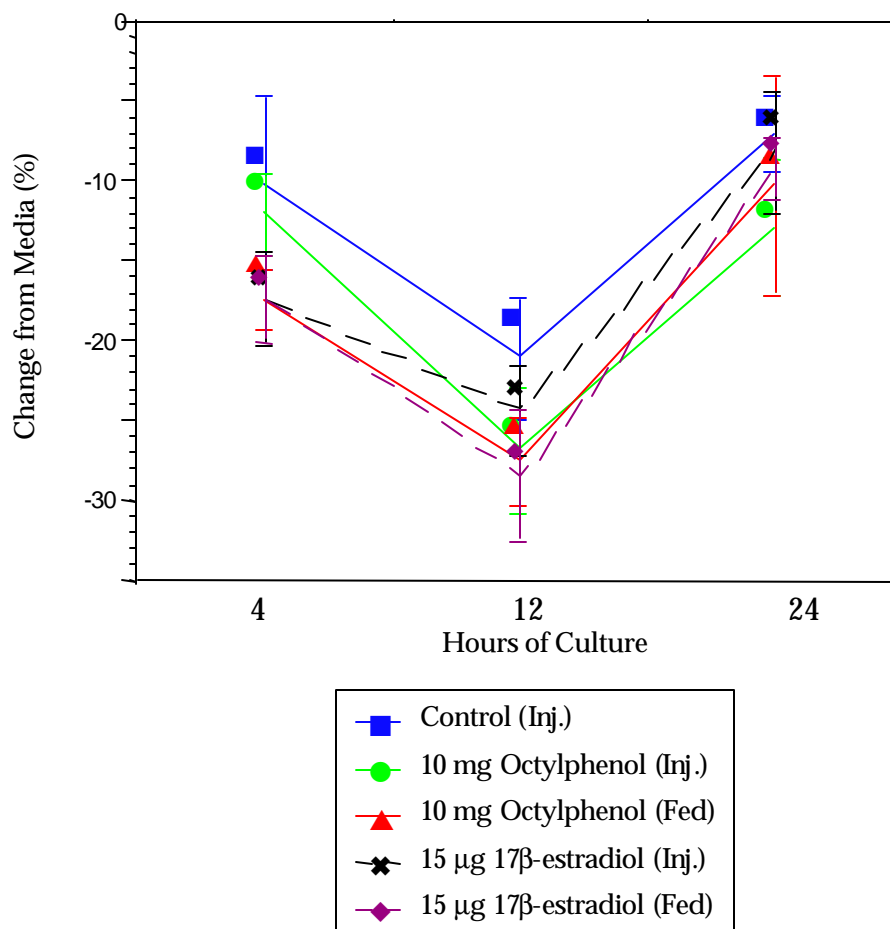
### Effect of Octylphenol Treatment in B/W Mice on the Apoptosis and Necrosis of Thymocytes upon PMA+I Stimulation



**Fig. 4.10:** Eight to ten-week old B/W males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15 µg 17β-estradiol, oral dosing of 10 mg of octylphenol or 15 µg of 17β-estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for 10 mg octylphenol injected at 4, 12, and 24 hours and 17β-estradiol injected at 12 hours; n=4 for all other groups and time points)

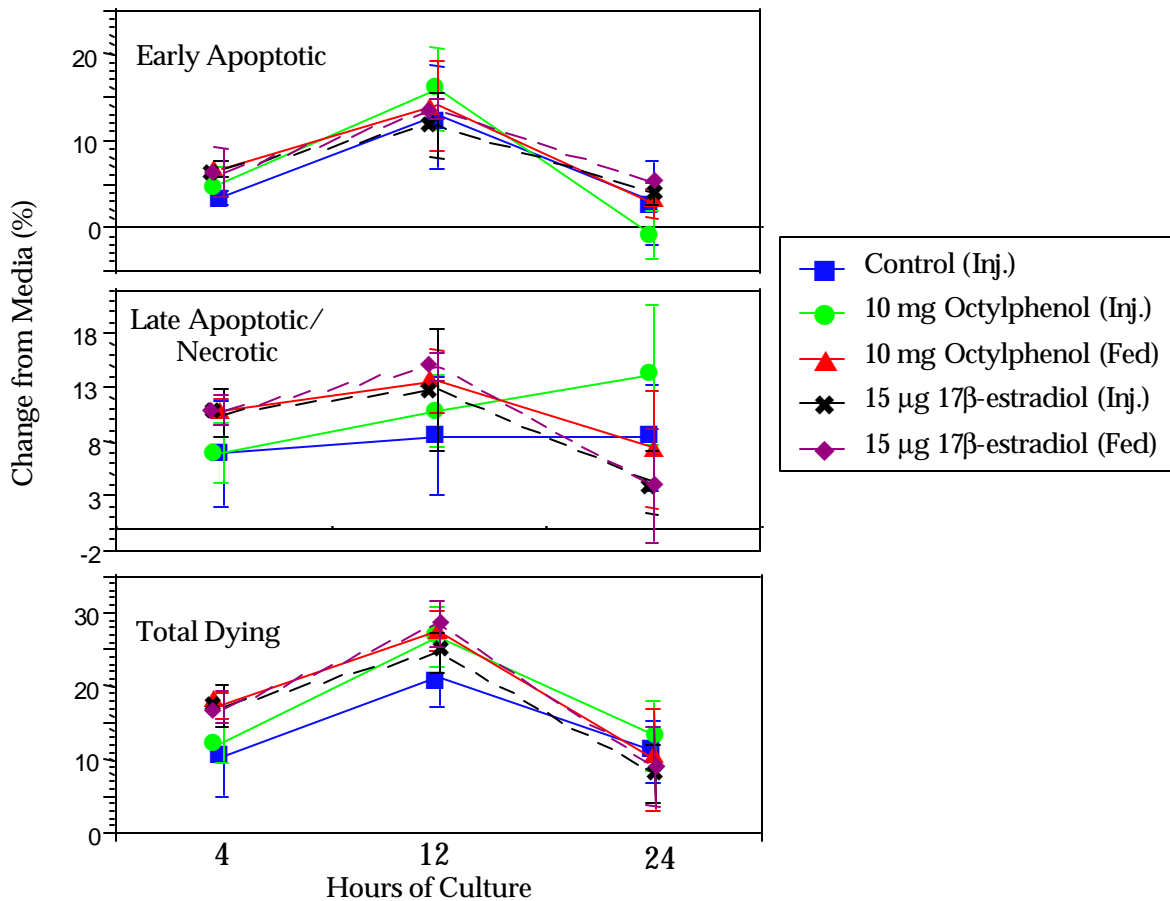


**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment in C57BL/6 Mice on the Proportion of Live (7-AAD<sup>dull</sup>) Thymocytes upon PMA+I Stimulation**



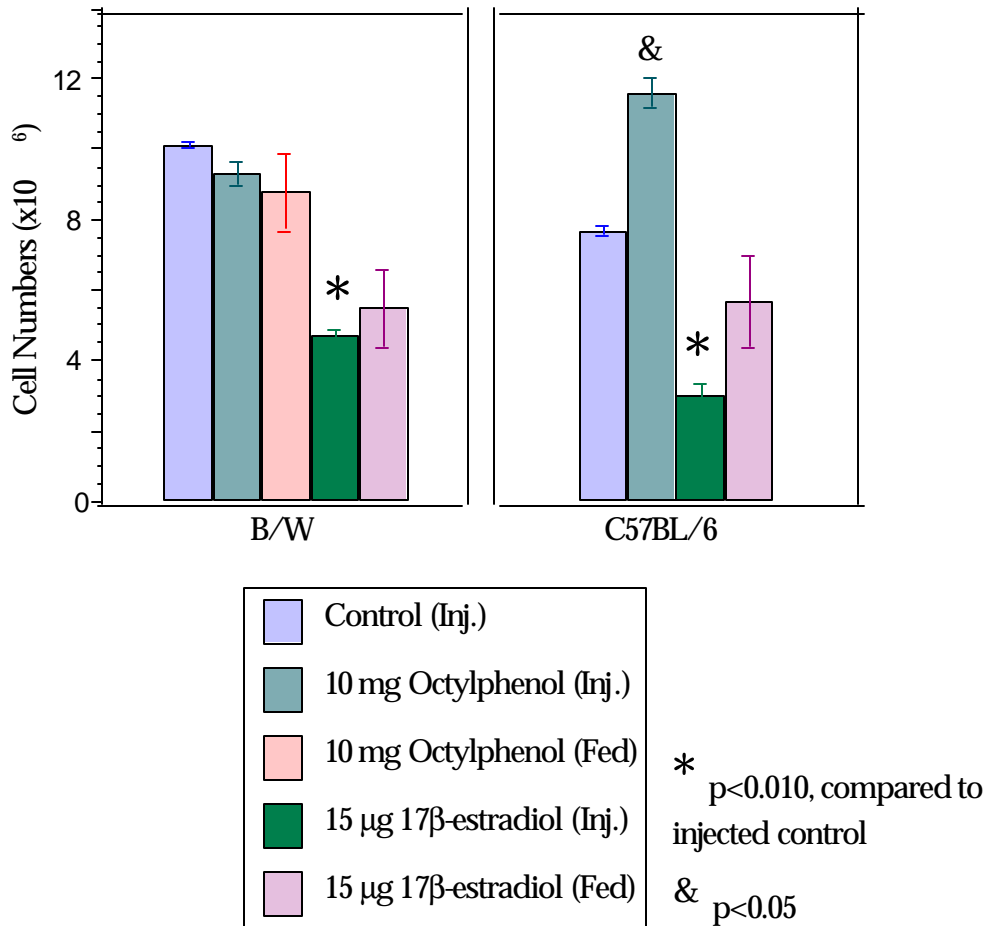
**Fig. 4.11:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in the proportion of 7-AAD<sup>dull</sup> (live) thymocytes (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for control, n=4 for 10 mg octylphenol injected, 10 mg octylphenol fed, and 17 $\beta$ -estradiol fed; n=5 for 17 $\beta$ -estradiol injected)

### Effect of Octylphenol Treatment in C57BL/6 Mice on the Apoptosis and Necrosis of Thymocytes upon PMA+I Stimulation



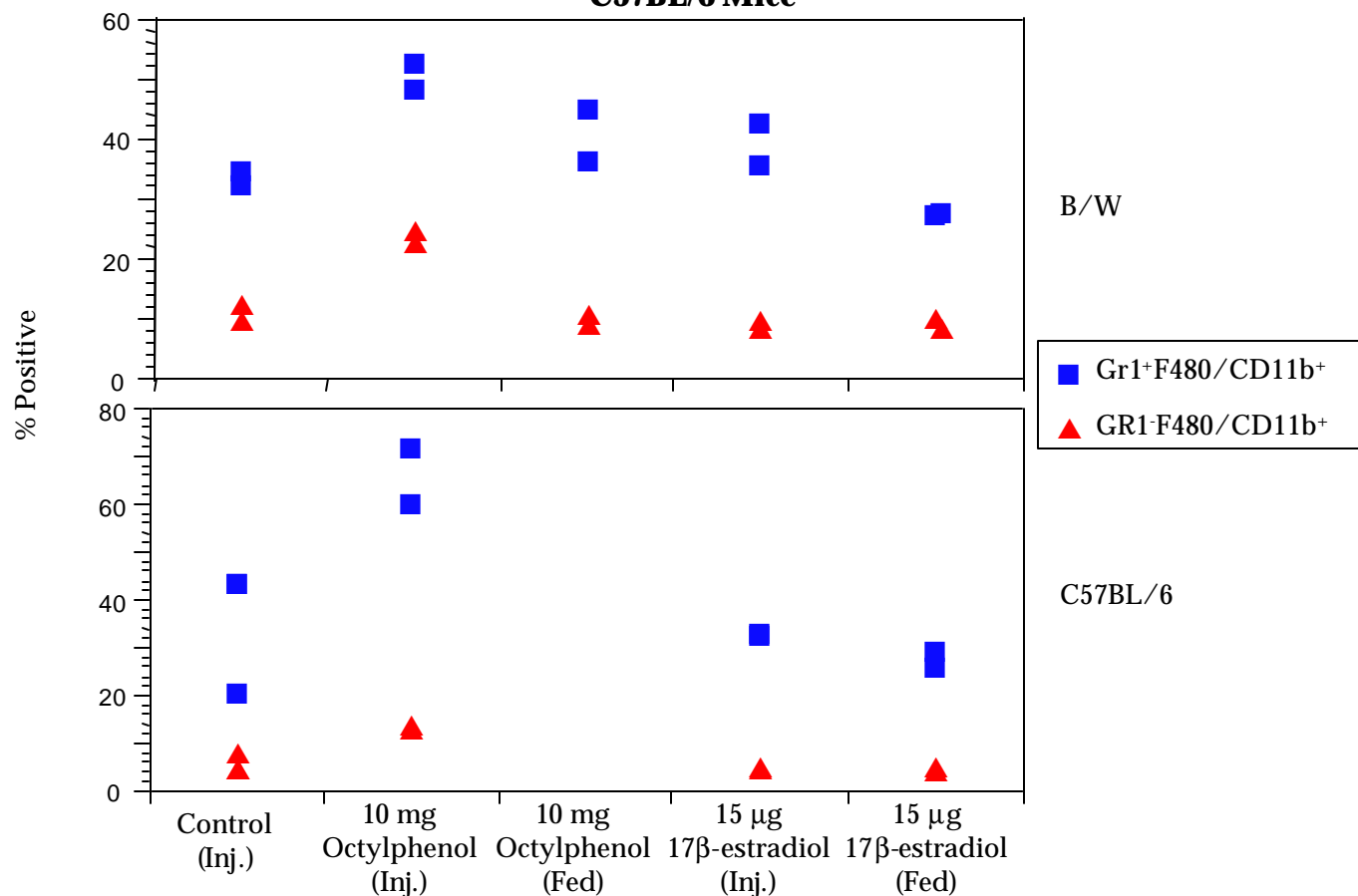
**Fig. 3.12:** Eight to ten-week old C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15 µg 17β-estradiol, oral dosing of 10 mg of octylphenol or 15 µg of 17β-estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the thymus was removed from both strains and the thymocytes were isolated. The thymocytes were stimulated with PMA+I or media for a baseline. At 4, 12, and 24 hours of culture the thymocytes were stained with 7-AAD and analyzed using an EPICS-XL flow cytometer to detect apoptosis. The data are represented as the change from the media baseline of the PMA+I stimulated thymocytes in each of the 7-AAD<sup>intermediate</sup> (early apoptotic), 7-AAD<sup>bright</sup> (late apoptotic/necrotic), and 7-AAD<sup>intermediate+bright</sup> (total dying) proportions of the population (% stimulated - % media). Media baselines are listed in the appendix.  $p \leq 0.010$  was considered significant. (n=3 for control, n=4 for 10 mg octylphenol injected, 10 mg octylphenol fed, and 17β-estradiol fed; n=5 for 17β-estradiol injected)

### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Cellularity of the Bone Marrow in B/W and C57BL/6 Mice



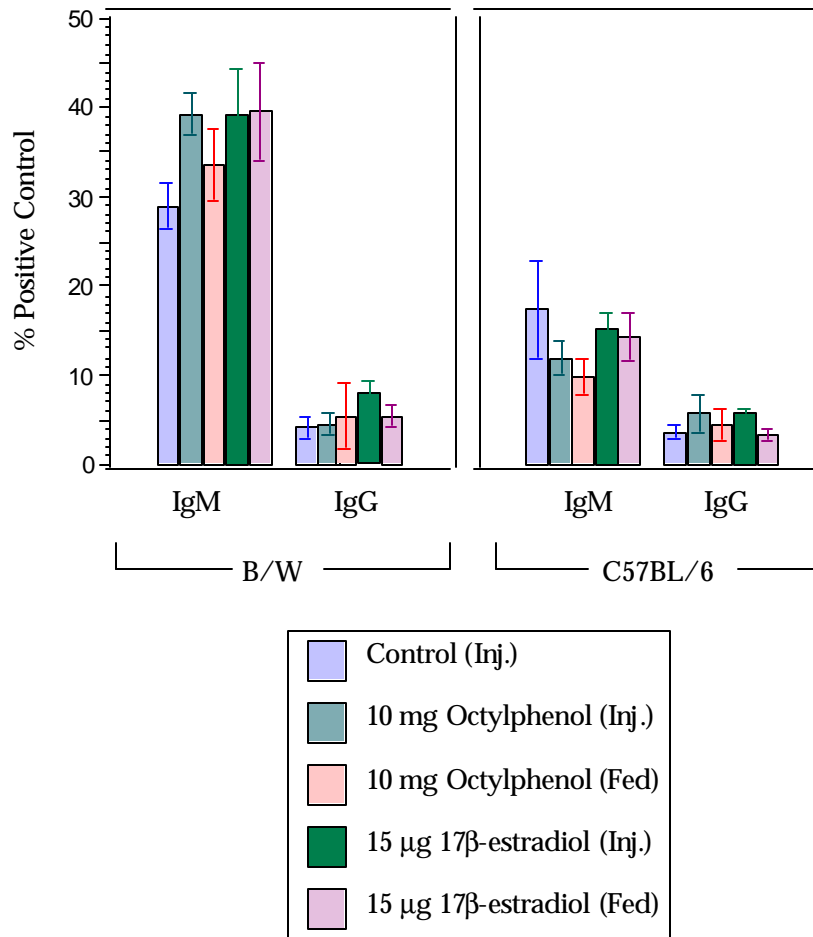
**Fig. 4.13:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the bone marrow was washed from the left femur of both strains, the red blood cells were removed, and the remaining cells were counted.  $p \leq 0.010$  was considered significant. (C57BL/6: n=2 for 10 mg octylphenol injected, 17 $\beta$ -estradiol injected, and 17 $\beta$ -estradiol fed; n=3 for control; B/W: n=2 for all groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Proportion of Developing Granulocytes, Macrophages, and Monocytes in the Bone Marrow of B/W and C57BL/6 Mice**



**Fig. 4.14:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the bone marrow was washed from the left femur of both strains and the cells were stained with PE-conjugated anti-GR1 and FITC-conjugated anti-F480 plus FITC-conjugated anti-CD11b. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented in dot plots as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=3 for control; n=2 for all other groups; B/W: n=1 for 17 $\beta$ -estradiol fed; n=2 for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Level of Anti-dsDNA Autoantibodies in the Serum of B/W and C57BL/6 Mice**



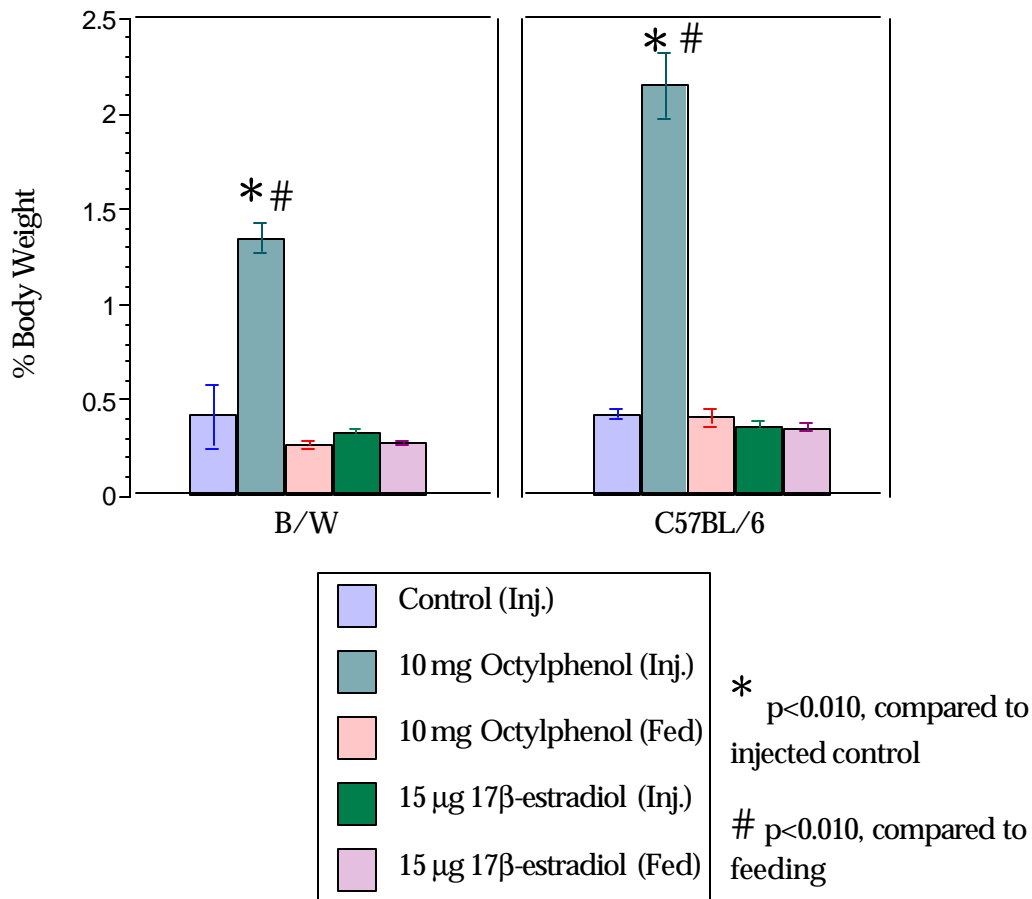
**Fig. 4.15:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment both strains were bled retro-orbitally and the serum removed. ELISAs were performed to determine the level of IgM and IgG anti-dsDNA autoantibodies in the serum. The data are presented as the percentage of the positive control serum to control for plate to plate variation.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected and 17 $\beta$ -estradiol fed; n=6 for all other groups)

**Table 4.1****Subcutaneous Injection of 10 mg of Octylphenol Results in Hepatic Hematopoiesis in B/W and C57BL/6 Mice**

	B/W		C57BL/6	
	Incidence	Percent	Incidence	Percent
Control	0/6	0	0/6	0
Octylphenol (Injection)	5/5	100	6/6	100
Octylphenol (Feeding)	0/6	0	1/5	20
17 $\beta$ -estradiol (Injection)	0/6	0	0/7	0
17 $\beta$ -estradiol (Feeding)	0/6	0	1/7	14

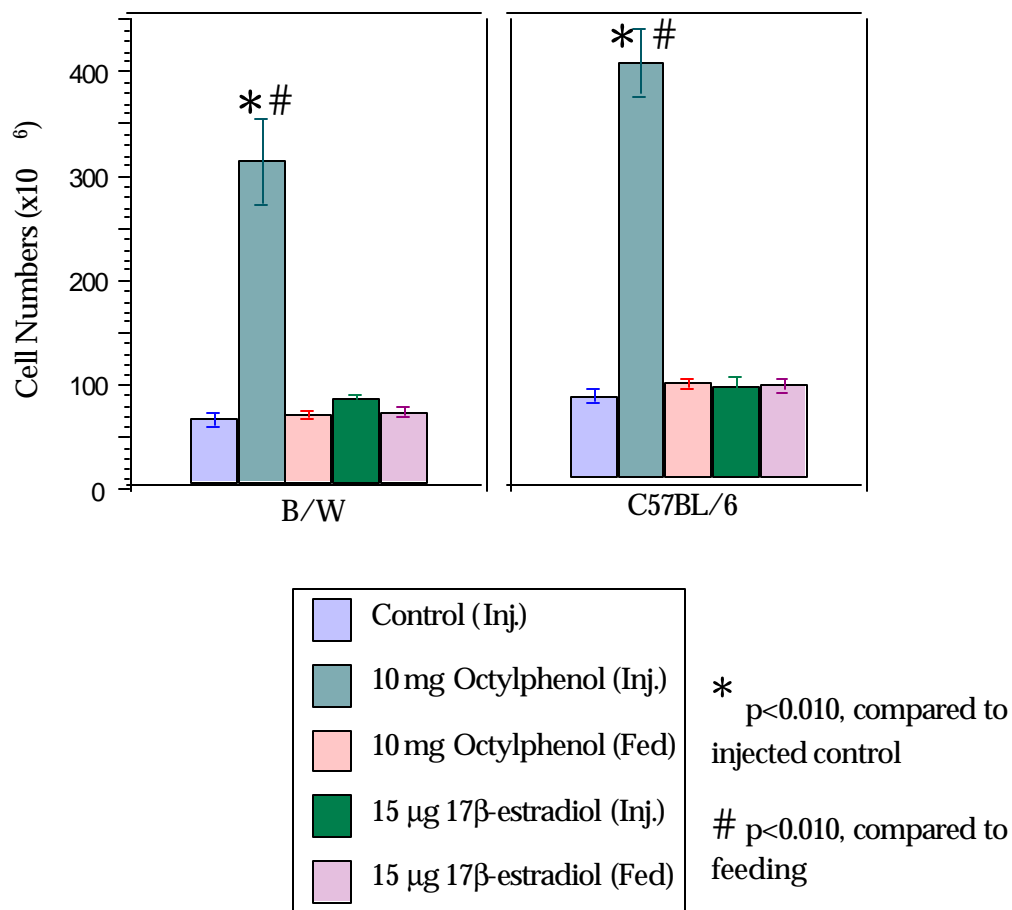
Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the liver was removed and fixed in 10% buffered formalin. Sections were examined by a board-certified pathologist

### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Spleen in B/W and C57BL/6 Mice



**Fig. 4.16:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment both strains were weighed and the spleen was removed. The spleen was weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6:  $n=5$  for 10 mg octylphenol fed;  $n=6$  for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed;  $n=7$  for 17 $\beta$ -estradiol fed; B/W:  $n=5$  for 10 mg octylphenol injected;  $n=6$  for all other groups)

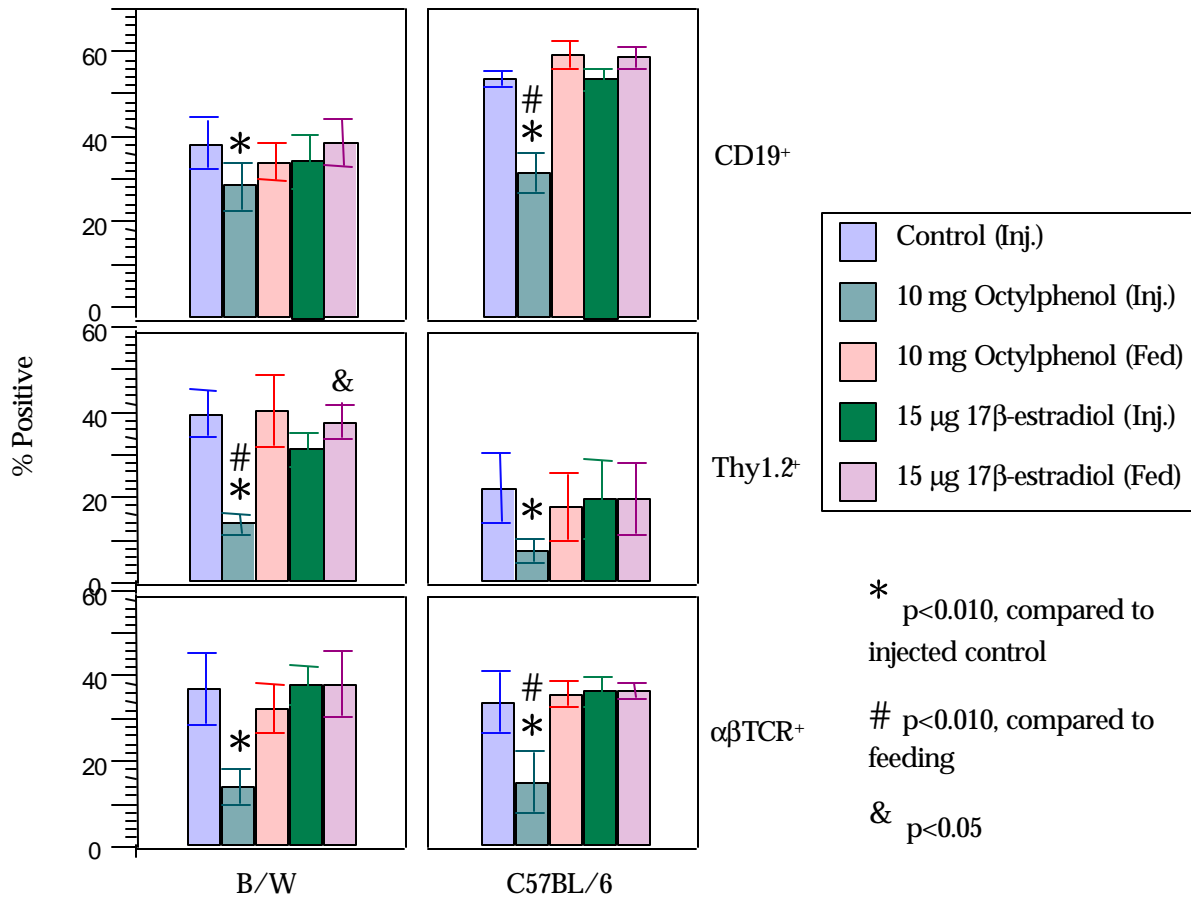
### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Leukocyte Cellularity of the Spleen in B/W and C57BL/6 Mice



**Fig. 4.17:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the spleen was removed from both strains. The leukocytes were isolated and counted.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected and 17 $\beta$ -estradiol fed; n=6 for all other groups)

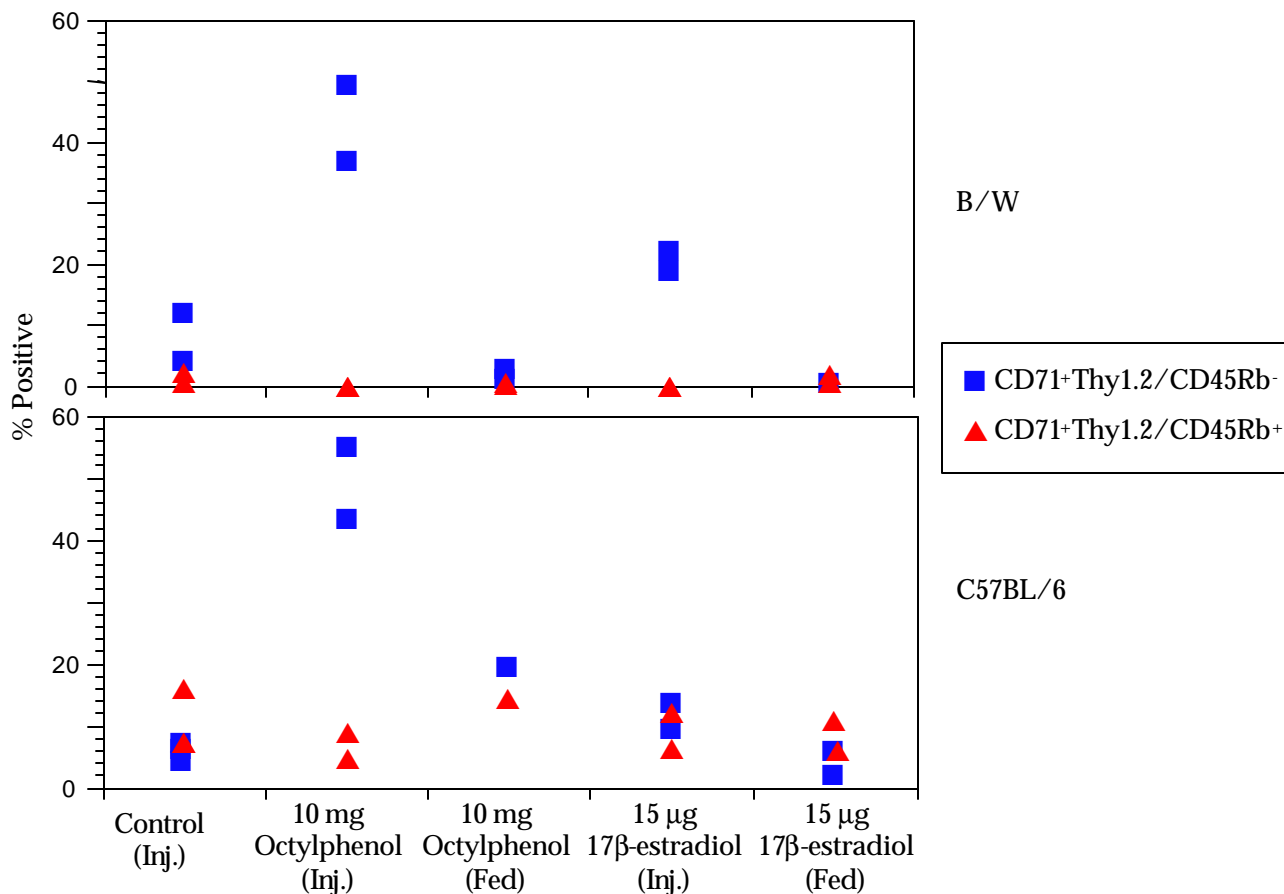


**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the T and B Cell Markers of Splenic Leukocytes in B/W and C57BL/6 Mice**



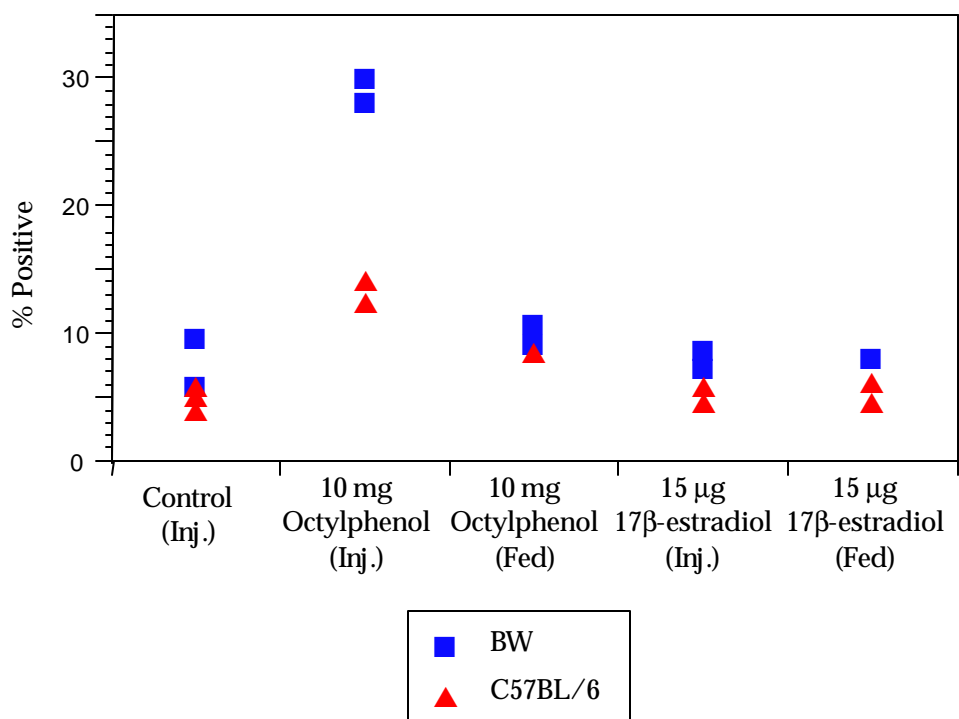
**Fig. 4.18:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the spleen was removed from both strains. The leukocytes were isolated from the spleen and stained with PE-conjugated anti-CD19 antibodies and FITC-conjugated anti-Thy1.2 antibodies, cy-chrome-conjugated anti- $\alpha\beta$ TCR antibodies, or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected and 17 $\beta$ -estradiol fed; n=6 for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Expression of CD71 on T and B cells from the Spleen of B/W and C57BL/6 Mice**



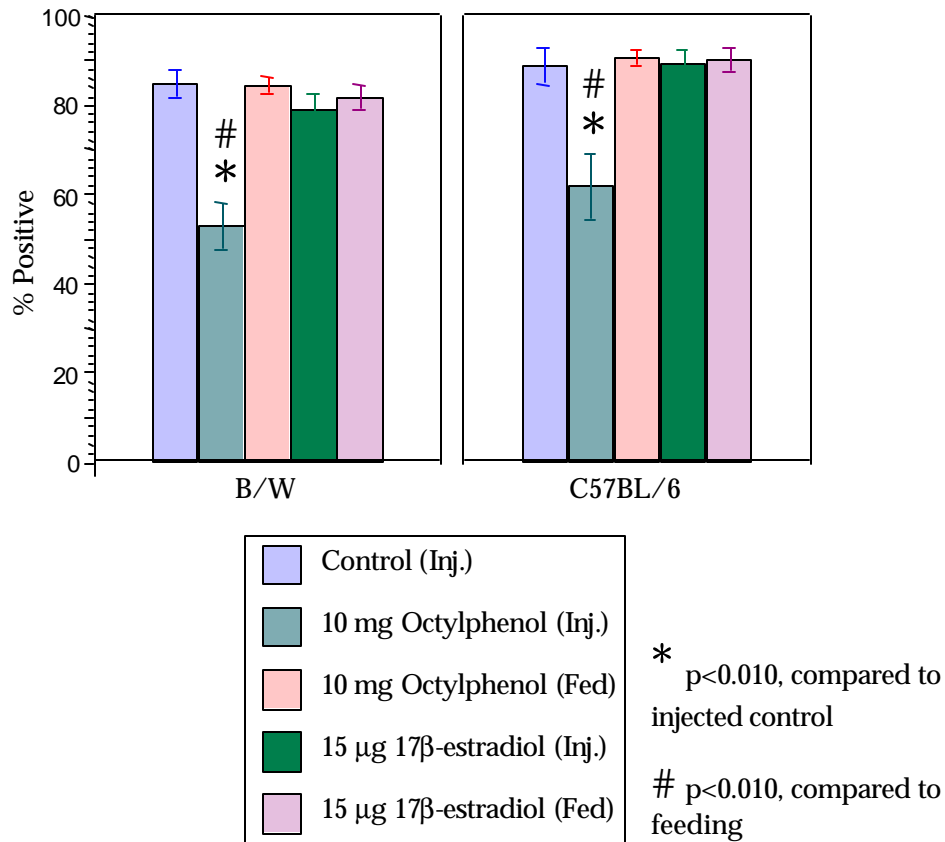
**Fig. 4.19:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the spleen was removed from both strains. The leukocytes were isolated from the spleen and stained with PE-conjugated anti-CD71 antibodies and FITC-conjugated anti-Thy1.2 antibodies plus FITC-conjugated anti-CD45Rb or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented in dot plots as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=1 for 10 mg octylphenol fed; n=2 for octylphenol injected, 17 $\beta$ -estradiol injected, and 17 $\beta$ -estradiol fed; n=3 for control; B/W: n=1 for 17 $\beta$ -estradiol fed; n=2 for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Proportion of GR1<sup>+</sup>F480/CD11b<sup>+</sup> Developing Granulocytes, Macrophages, and Monocytes in the Spleen of B/W and C57BL/6 Mice**



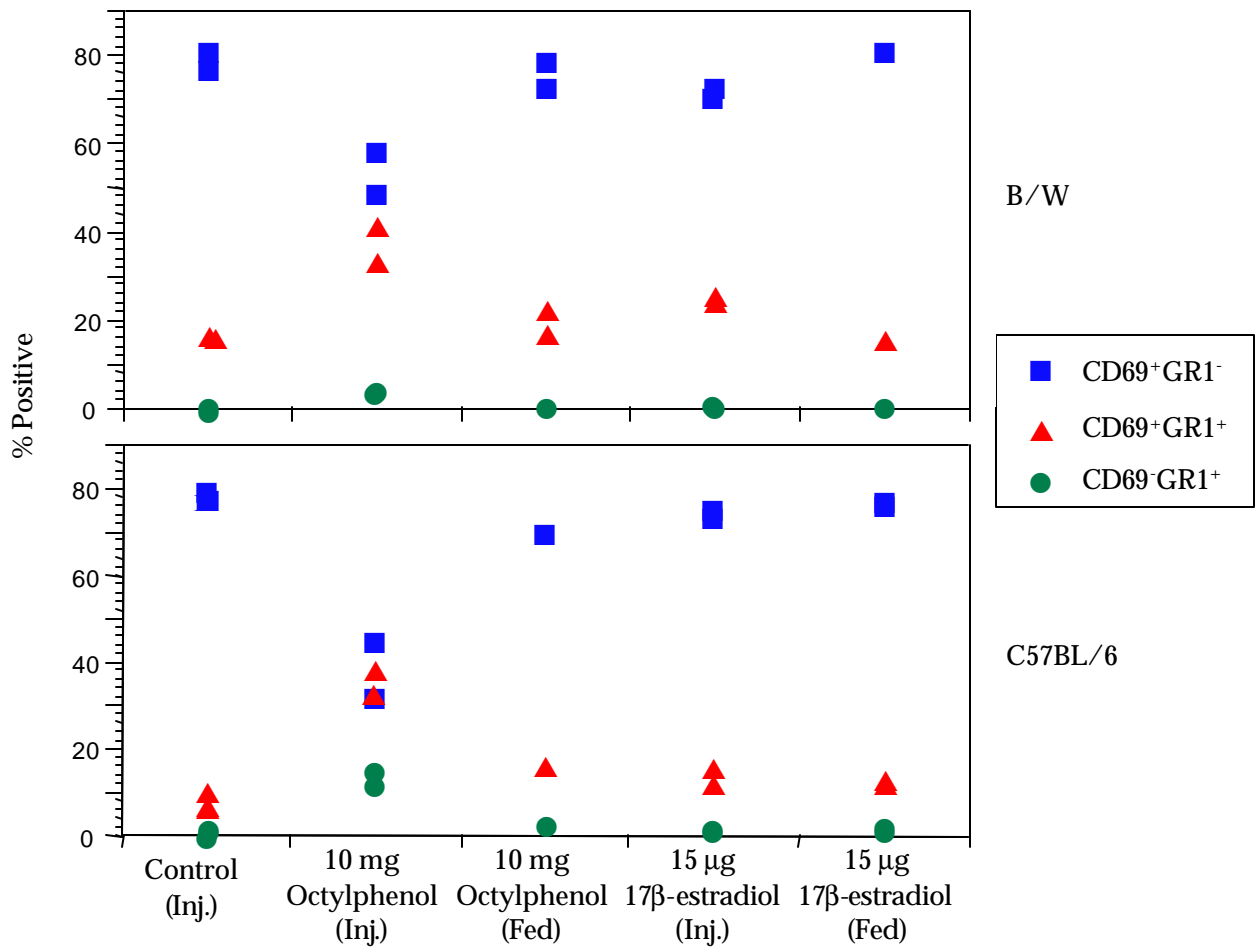
**Fig. 4.20:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the spleen was removed from both strains. The leukocytes were isolated from the spleen and stained with PE-conjugated anti-GR1 antibodies and FITC-conjugated anti-F480 plus FITC-conjugated anti-CD11b antibodies or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented in dot plots as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=1 for 10 mg octylphenol fed; n=2 for octylphenol injected, 17 $\beta$ -estradiol injected, and 17 $\beta$ -estradiol fed; n=3 for control; B/W: n=1 for 17 $\beta$ -estradiol fed; n=2 for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Expression of Very Early Activation Antigen, CD69, on Splenic Leukocytes from B/W and C57BL/6 Mice**



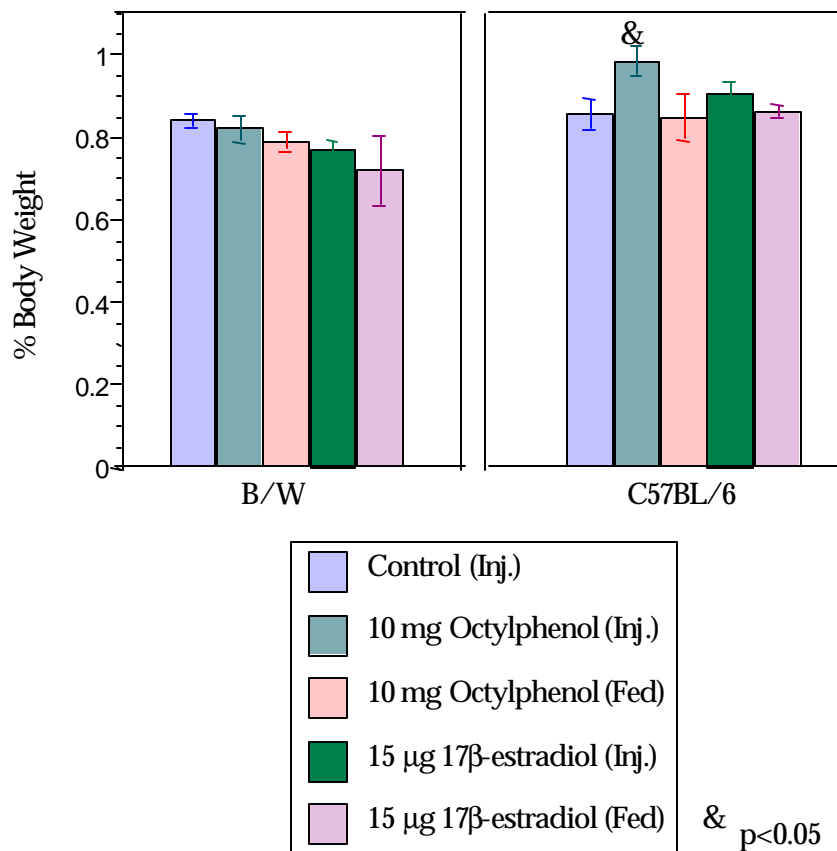
**Fig. 4.21:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and lymph nodes and stimulated with PMA+I. After 4 hours of culture the cells were stained with PE-conjugated anti-CD69 or an isotype matched control. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected and 17 $\beta$ -estradiol fed; n=6 for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Expression of CD69 and GR1 on Splenic Leukocytes from B/W and C57BL/6 Mice**



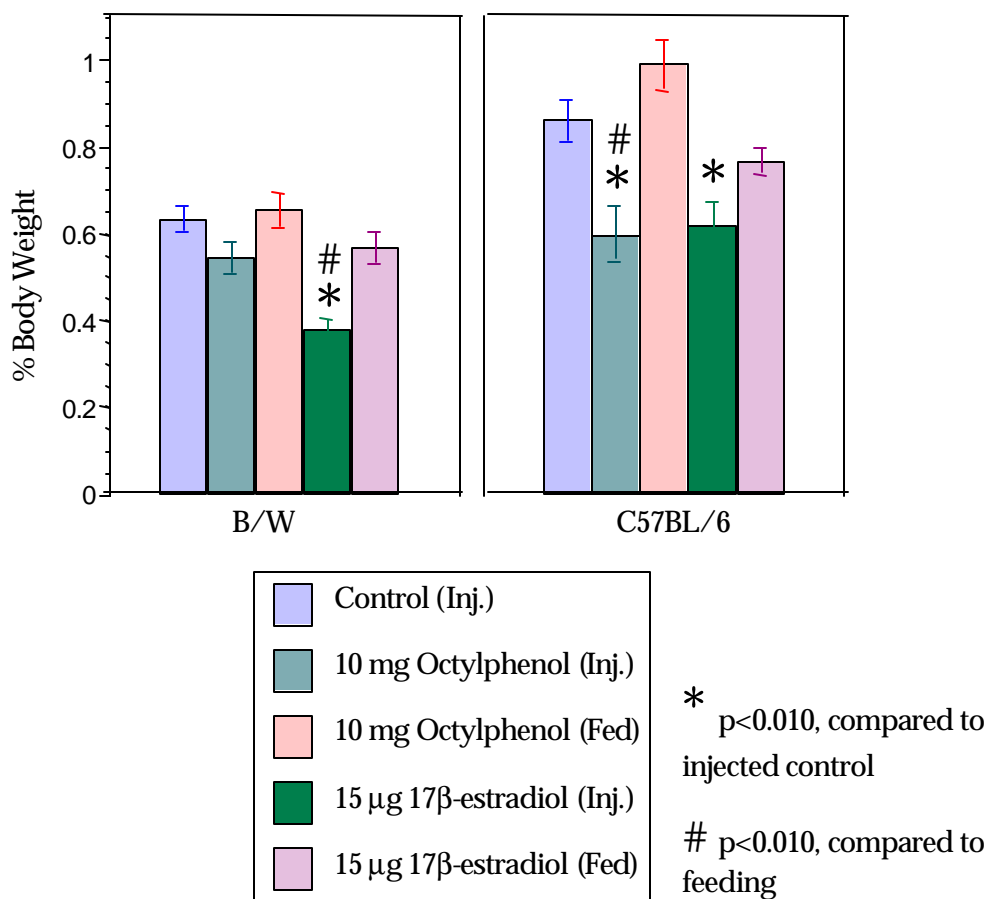
**Fig. 4.22:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment the leukocytes were isolated from the spleen and stimulated with PMA+I. After 4 hours of culture the cells were stained with FITC-conjugated anti-CD69 and PE-conjugated anti-GR1 or isotype matched controls. The stained cells were analyzed using an EPICS-XL flow cytometer. The data are presented in dot plots as the percentage of cells that stained positive.  $p \leq 0.010$  was considered significant. (C57BL/6: n=1 for 10 mg octylphenol fed; n=2 for octylphenol injected, 17 $\beta$ -estradiol injected, and 17 $\beta$ -estradiol fed; n=3 for control; B/W: n=1 for 17 $\beta$ -estradiol fed; n=2 for all other groups)

**Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Testis in B/W and C57BL/6 Mice**



**Fig. 4.23:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment both strains were weighed and the testis were removed. The testis were weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected and 17 $\beta$ -estradiol fed; n=6 for all other groups)

### Effect of Octylphenol and 17 $\beta$ -estradiol Treatment on the Percent Body Weight of the Seminal Vesicles in B/W and C57BL/6 Mice



**Fig. 4.24:** Eight to ten-week old B/W and C57BL/6 males were treated three times a week for three weeks with subcutaneous injections of 10 mg of octylphenol or 15  $\mu$ g 17 $\beta$ -estradiol, oral dosing of 10 mg of octylphenol or 15  $\mu$ g of 17 $\beta$ -estradiol, or subcutaneous injection of an ethanol/oil control. Three days after the last treatment both strains were weighed and the seminal vesicles were removed. The seminal vesicles were weighed and the percent body weight was calculated.  $p \leq 0.010$  was considered significant. (C57BL/6: n=5 for 10 mg octylphenol fed; n=6 for control, 10 mg octylphenol injected, and 17 $\beta$ -estradiol fed; n=7 for 17 $\beta$ -estradiol fed; B/W: n=5 for 10 mg octylphenol injected; n=6 for all other groups)

## Chapter 5

### General Discussion

The list of endocrine disrupting chemicals in the environment that are able to mimic estrogen is very long and continually growing. These chemicals are classified as endocrine disrupting chemicals because their ability to mimic estrogen in the reproductive tissue allows them to disrupt the endocrine system. It is well established that estrogen is able to modulate the immune system (Grossman, 1984; Ansar Ahmed et al., 1985a; Olsen and Kovacs, 1996). This opens the possibility that any endocrine disrupting chemical that is able to mimic estrogen may be able to modulate the immune system. The increasing list of chemicals that are able of mimic estrogen and the large number of models, depending on age, gender, and route of administration, in which to test them leaves a vast amount of work to be done to determine whether we should be concerned about the ability of endocrine disrupting chemicals to modulate the immune system.

The main purpose of these studies was to determine the effects of genistein and octylphenol on the immune system of non-autoimmune and genetically prone-autoimmune mice. However, these studies were only able to closely examine the effects of octylphenol on the thymus of reproductively intact, mature C57BL/6 and B/W males. The preliminary study in Chapter 2 showed us that the thymus of orchietomized B/W males and, possibly, IFN- $\gamma$  secretion by orchietomized B/W males is affected by oral octylphenol treatment; and that the weight of the thymus and spleen are affected by genistein treatment. The study in Chapter 3 examined a range of doses of octylphenol, and showed that the thymus of reproductively mature, intact C57BL/6 and B/W males (unlike orchietomized B/W males) is not affected by the same or lower oral doses of octylphenol. However, octylphenol also did not show estrogenic changes in the reproductive tract, and the 17 $\beta$ -estradiol positive control did not have all the estrogenic effects expected. The study in Chapter 4 was conducted to ensure the findings in the previous study were valid. The effects of subcutaneously injected octylphenol on the thymus and the reproductive tract was examined to determine whether octylphenol is estrogenic in our model, as it has been shown to be in other models (Blake and Boockfor, 1997; Boockfor and Blake, 1997). Also, the effects of subcutaneous injection and feeding were compared for octylphenol and 17 $\beta$ -estradiol to determine the importance of the route of exposure in the estrogenic potency of both chemicals. Due to the induction of inflammatory



dermal lesions by subcutaneous injection of octylphenol, it was not possible to ascertain its estrogenic effects or the importance of route of exposure for estrogenic potency. Observations on the effects of injection and feeding of 17 $\beta$ -estradiol showed that oral dosing, not our handling, decreased the activity of the compound. In summation, the main conclusion to draw from these studies about the effects of octylphenol on the immune system is that short-term oral dosing of 10 mg, 1 mg, or 0.1 mg of octylphenol does not affect the morphology or function of the thymus in reproductively mature, intact C57BL/6 or B/W males.

The route of exposure and the model in which the endocrine disrupting chemicals were tested were two very important variables when assessing the immunomodulatory effects and the relevance of these studies. The route of exposure may determine the estrogenic activity of an EDC. Different routes of exposure result in varying levels of exposure of diverse target organs. Moreover, the differing routes of administration may result in different metabolic and detoxification pathways. The studies in chapters 3 and 4 exhibit the importance of the route of exposure in an effective estrogenic dose of 17 $\beta$ -estradiol. Oral dosing of 17 $\beta$ -estradiol seems to decrease its estrogenic activity, and, therefore, a much higher dose was necessary in order to see the estrogenic effects seen with subcutaneous exposure. Endocrine disrupting chemicals are generally weakly estrogenic and the route of exposure may be important in determining whether an environmentally relevant dose has estrogenic activity upon exposure. However, if the purpose of the study is to determine whether to be concerned about environmental exposure to EDCs, then relevant routes of exposure and environmental doses become pivotal factors.

Furthermore, all the studies exhibit the importance of the model in which the chemical is tested. Chapters 3 and 4 show that chemicals may have different effects in different strains of mice. For example, data in both chapter 3 and chapter 4 suggest that the thymus is more sensitive to oral estrogenic effects than the reproductive tract in reproductively mature B/W males, and that the reproductive tract is more sensitive to oral estrogenic effects than the thymus in reproductively mature C57BL/6 males. Furthermore, chapters 2 and 3 exhibit the importance of the level of endogenous hormones in the model on the effects of the weak environmental estrogens. In orchietomized B/W males, octylphenol significantly decreases the thymic cellularity; whereas, in intact B/W males, the thymus is not affected by the same octylphenol treatment. As discussed earlier in chapter 3, the endocrine system is highly regulated by a feedback system and hormone levels are interdependent because of the feedback

regulation and because testosterone can be converted into estrogen. Furthermore, testosterone has been shown to ameliorate the estrogen-induced acceleration of autoimmune disease in B/W females (Melez et al, 1978). The endogenous levels of testosterone in the intact males utilized in the studies in chapter 3 may have interfered in the induction of thymic atrophy or other estrogenic effects. Therefore, it is important to determine a relevant model in which to test an EDC and to be cautious when extrapolating findings to other strains, and especially other species.

Possible exposure of the laboratory animals or *in vitro* cells to other endocrine disrupting chemicals is also an important variable to consider. Many commercially available murine diets are high in phytoestrogens and exposure of the laboratory animals through the feed may interfere in the testing of the weak EDCs (Thigpen et al, 1999). Furthermore, when testing EDCs *in vitro* or culturing cells *in vitro* after *in vivo* exposure to EDCs, exposure to other EDCs through plasticware is also possible (Soto et al, 1991). p-Nonylphenol has been shown to be released from the polystyrene of plasticware and has been shown to interfere with the results of EDC testing (Soto et al, 1991). Therefore, exposure to other EDCs throughout the experiments is another important variable to consider.

Modulation of the immune system by EDC could vary with the age and particular strain of mice. Prenatal or neonatal mice whose immune systems are still developing and may be sensitive to estrogenic effects, or older mice of an autoimmune strain to determine whether the onset of disease can be accelerated by octylphenol. Female mice of different ages could also be studied to determine whether different level of endogenous estrogen result in differing effects of octylphenol. Furthermore, dermal exposure can be examined. Dermal exposure is a relevant route of exposure for octylphenol, and it may have different effects from oral exposure. Our study found that oral octylphenol treatment did not affect the thymus of reproductively mature B/W or C57BL/6 males, however, different models, doses, or routes of exposure may have very different results. Furthermore, octylphenol could have caused subtle immunological effects that could not be detected in our assays. It is possible that subtle changes could affect the ability of octylphenol-treated mice to respond to infection or handle the onset of neoplastic conditions. This is another avenue in which octylphenol treatment needs to be tested.

Furthermore, future studies need to examine further the effects of octylphenol on the secretion of IFN- $\gamma$  by lymphocytes from secondary lymphoid organs. The preliminary studies suggested that lymphocytes from the spleen and lymph nodes of octylphenol treated mice had a decreased ability to secrete

IFN- $\gamma$  upon mitogen stimulation. A decrease in IFN- $\gamma$  secretion could have deleterious effects on the functioning of the immune system. It may cause a decrease in the ability of the immune system to initiate an immune response because the IFN- $\gamma$  may not be present to induce macrophages and natural killer cells to secrete more IL-12 and IL-18. Furthermore, IFN- $\gamma$  secretion is utilized in T helper 1 cytotoxic responses to intracellular infections. A decrease in IFN- $\gamma$  secretion could lead to a T helper 2, B cell mediated responses, which would be ineffective against an intracellular pathogen (Kasuga-Aoki et al, 1999). Furthermore, IFN- $\gamma$  inhibits apoptosis of malignant, CD5+ B lymphocytes of B cell chronic lymphocytic leukemia; therefore, a decrease in IFN- $\gamma$  could affect the progression of the leukemia or possibly other diseases (Buschle et al, 1993). Studies need to confirm whether or not oral octylphenol can affect mitogen-induced or antigen-specific stimulation of IFN- $\gamma$  secretion in reproductively intact, mature males or in any other model. If IFN- $\gamma$  is affected by octylphenol treatment in any model, the consequences of this decrease on the function of the immune system needs to be examined.

There are many endocrine disrupting chemicals in the environment that are able to mimic estrogen and, therefore, may modulate the immune system, and the list is growing. There are also many combinations of age, gender, and route of exposure that can be examined for each chemical, not taking into account the many different strains of mice, rats, or species of lab animal in which to study them. This is one small initial study in the expanse of studies that need to be conducted to definitively determine the level of concern necessary for the ability of endocrine disrupting chemicals in our environment to modulate the immune system.

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## Appendix

### Media Baselines

Fig 2.3: The media baseline fluorescence averaged  $1380.0 \pm 66.7$  at 8 hours and  $1700.9 \pm 60.4$  at 24 hours for control mice and  $1469.6 \pm 131.8$  at 8 hours and  $1765.6 \pm 111.8$  at 24 hours for octylphenol treated mice.

Fig 2.4: The proportion of cells within each category for the media baseline are as follows: 8 hours Control - sub Go  $4.78 \pm 1.53$ , Go/G1  $87.08 \pm 2.36$ , S/G2/M  $5.85 \pm 0.95$ ; Octylphenol - sub Go  $10.6 \pm 2.22$ , Go/G1  $79.48 \pm 3.02$ , S/G2/M  $7.2 \pm 0.55$ ; 24 hours Control - sub Go  $44.7 \pm 9.93$ , Go/G1  $47.93 \pm 11.81$ , S/G2/M  $1.7 \pm 0.32$ ; Octylphenol - sub Go  $43.9 \pm 8.24$ , Go/G1  $47.44 \pm 9.31$ , S/G2/M  $1.94 \pm 0.39$ .

Fig 2.10: The media baseline fluorescence for lymphocytes from the spleen averaged  $1018.2 \pm 205.5$  for control mice and  $1100.1 \pm 58.7$  for octylphenol treated mice at 8 hours,  $1411.0 \pm 147.5$  for control mice and  $1332.8 \pm 74.9$  for octylphenol treated mice at 24 hours, and  $1892.9 \pm 213.4$  for control mice and  $1735.3 \pm 78.1$  for octylphenol treated mice at 48 hours. The media baseline fluorescence for lymphocytes from the lymph nodes averaged  $666.7 \pm 0$  for control mice and  $786.2 \pm 30.7$  for octylphenol treated mice at 12 hours,  $1156.7 \pm 160.7$  for control mice and  $1026.3 \pm 21.6$  for octylphenol treated mice at 24 hours, and  $1555.5 \pm 224.6$  for control mice and  $1336.3 \pm 31.8$  for octylphenol treated mice at 48 hours.

Fig. 2.11: The proportion of cells within each category for the media baseline are as follows: 24 hours Control - sub Go  $50.4 \pm 1.36$ , Go/G1  $42.75 \pm 1.86$ , S/G2/M  $3.03 \pm 0.2$ ; Octylphenol - sub Go  $53.9 \pm 1.82$ , Go/G1  $38.38 \pm 2.37$ , S/G2/M  $3.35 \pm 0.28$ ; 48 hours Control - sub Go  $58.0 \pm 1.03$ , Go/G1  $35.88 \pm 1.61$ , S/G2/M  $3.25 \pm 0.36$ ; Octylphenol - sub Go  $59.0 \pm 2.42$ , Go/G1  $35.62 \pm 3.15$ , S/G2/M  $3.18 \pm 0.33$ .

Fig. 2.12: The proportion of cells within each category for the media baseline are as follows: 24 hours Control - sub Go  $52.6 \pm 1.55$ , Go/G1  $37.6 \pm 1.3$ , S/G2/M  $6.5 \pm 0.1$ ; Octylphenol - sub Go  $61.0 \pm 1.2$ , Go/G1  $28.7 \pm 1.9$ , S/G2/M  $5.11 \pm 0.05$ ; 48 hours Control - sub Go  $50.6 \pm 9.52$ , Go/G1  $40.33 \pm 12.63$ , S/G2/M  $5.20 \pm 1.36$ ; Octylphenol - sub Go  $53.2 \pm 7.4$ , Go/G1  $38.54 \pm 7.74$ , S/G2/M  $3.82 \pm 0.61$ .

Fig 2.15: The media baseline fluorescence averaged  $1168.5 \pm 76.7$  at 12 hours and  $1403.5 \pm 94.7$  at 24 hours for control mice and  $1229.7 \pm 89.6$  at 12 hours and  $1390.8 \pm 84.7$  at 24 hours for genistein treated mice.

Fig. 2.16: The proportion of cells within each category for the media baseline are as follows: 12 hours Control - sub Go  $56.2 \pm 5.2$ , Go/G1  $31.8 \pm 7.4$ , S/G2/M  $8.2 \pm 0.8$ ; Genistein - sub Go  $59.0 \pm 0.7$ , Go/G1  $29.5 \pm 3.1$ , S/G2/M  $7.6 \pm 0.8$ ; 24 hours Control - sub Go  $67.6 \pm 1.5$ , Go/G1  $20.4 \pm 5.1$ , S/G2/M  $7.8 \pm 1.9$ ; Genistein - sub Go  $62.1 \pm 2.5$ , Go/G1  $22.6 \pm 1.2$ , S/G2/M  $6.9 \pm 1.1$ .

Fig. 2.19: The media baseline fluorescence for lymphocytes from the spleen averaged  $996.9 \pm 80.1$  for control mice and  $1100.2 \pm 47.1$  for genistein treated mice at 12 hours,  $1028.3 \pm 69.2$  for control mice and  $1141.1 \pm 54.2$  for genistein treated mice at 24 hours, and  $1344.1 \pm 48.2$  for control mice and  $1457.5 \pm 60.3$  for genistein treated mice at 48 hours. The media baseline fluorescence for lymphocytes from the lymph nodes averaged  $974.7 \pm 9.4$  for control mice and  $851.9 \pm 75.7$  for genistein treated mice at 12 hours,  $1251.0 \pm 78.9$  for control mice and  $1069.3 \pm 32.0$  for genistein treated mice at 24 hours, and  $1542.7 \pm 112.5$  for control mice and  $1311.1 \pm 37.1$  for genistein treated mice at 48 hours.

Fig. 2.20: The proportion of cells within each category for the media baseline are as follows: 24 hours Control - sub Go  $40.0 \pm 5.3$ , Go/G1  $45.1 \pm 6.0$ , S/G2/M  $3.7 \pm 0.6$ ; Genistein - sub Go  $40.9 \pm 6.8$ , Go/G1  $45.1 \pm 7.0$ , S/G2/M  $2.9 \pm 0.3$ ; 48 hours Control - sub Go  $55.7 \pm 3.9$ , Go/G1  $36.8 \pm 3.8$ , S/G2/M  $3.6 \pm 1.0$ ; Genistein - sub Go  $52.9 \pm 2.5$ , Go/G1  $39.4 \pm 1.9$ , S/G2/M  $3.5 \pm 0.7$ .

Fig. 2.21: The proportion of cells within each category for the media baseline are as follows: 48 hours Control - sub Go  $58.6 \pm 3.5$ , Go/G1  $30.5 \pm 3.2$ , S/G2/M  $2.9 \pm 0.7$ ; Genistein - sub Go  $56.1 \pm 2.9$ , Go/G1  $31.5 \pm 2.4$ , S/G2/M  $4.4 \pm 0.6$ .

Fig. 3.5: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours -  $86.9 \pm 3.0$ - $92.1 \pm 1.0$ ; 12 hours -  $75.5 \pm 4.8$ - $86.7 \pm 0.6$ ; 24 hours -  $60.6 \pm 4.1$ - $76.1 \pm 0.8$ .

Fig 3.6: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hour - early apoptotic -  $3.9 \pm 0.6$ - $7.2 \pm 1.7$ , late apoptotic/necrotic -  $3.0 \pm 0.4$ - $5.2 \pm 1.3$ , total dying -  $7.1 \pm 0.9$ - $12.4 \pm 2.9$ ; 12 hour - early apoptotic -  $5.3 \pm 0.6$ - $11.9 \pm 1.7$ , late apoptotic/necrotic -  $7.2 \pm 0.3$ - $12.0 \pm 3.0$ , total dying -  $12.5 \pm 0.7$ - $23.8 \pm 4.7$ ; 24 hour - early apoptotic -  $7.2 \pm 0.5$ - $15.7 \pm 1.1$ , late apoptotic/necrotic -  $15.9 \pm 0.3$ - $22.9 \pm 3.5$ , total dying -  $23.3 \pm 0.7$ - $38.6 \pm 3.9$ .

Fig. 3.7: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours -  $81.7 \pm 6.6$ - $90.8 \pm 0.5$ ; 12 hours -  $72.3 \pm 7.0$ - $81.2 \pm 2.9$ ; 24 hours -  $51.7 \pm 6.7$ - $63.7 \pm 3.9$ ,

Fig 3.8: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hour - early apoptotic -  $4.3 \pm 0.2$ - $9.5 \pm 3.6$ , late apoptotic/necrotic -  $4.3 \pm 0.4$ - $8.1 \pm 2.9$ , total dying -  $8.6 \pm 0.5$ - $16.8 \pm 6.9$ ; 12 hour - early apoptotic -  $6.6 \pm 0.5$ - $12.7 \pm 3.6$ , late apoptotic/necrotic -  $11.6 \pm 1.7$ - $16.8 \pm 4.8$ , total dying -  $18.3 \pm 1.6$ - $27.1 \pm 7.1$ ; 24 hour - early apoptotic -  $11.9 \pm 1.5$ - $16.8 \pm 2.8$ , late apoptotic/necrotic -  $23.2 \pm 3.1$ - $30.2 \pm 4.2$ , total dying -  $35.6 \pm 4.0$ - $47.0 \pm 6.7$ .

Fig. 3.9: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours -  $86.9 \pm 3.0$ - $92.1 \pm 1.0$ ; 12 hours -  $75.5 \pm 4.8$ - $86.7 \pm 0.6$ ; 24 hours -  $60.6 \pm 4.1$ - $76.1 \pm 0.8$ .

Fig 3.10: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hour - early apoptotic -  $3.9 \pm 0.6$ - $7.2 \pm 1.7$ , late apoptotic/necrotic -  $3.0 \pm 0.4$ - $5.2 \pm 1.3$ , total dying -  $7.1 \pm 0.9$ - $12.4 \pm 2.9$ ; 12 hour - early apoptotic -  $5.3 \pm 0.6$ - $11.9 \pm 1.7$ , late apoptotic/necrotic -  $7.2 \pm 0.3$ - $12.0 \pm 3.0$ , total dying -  $12.5 \pm 0.7$ - $23.8 \pm 4.7$ ; 24 hour - early apoptotic -  $7.2 \pm 0.5$ - $15.7 \pm 1.1$ , late apoptotic/necrotic -  $15.9 \pm 0.3$ - $22.9 \pm 3.5$ , total dying -  $23.3 \pm 0.7$ - $38.6 \pm 3.9$ .

Fig. 3.11: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours -  $81.7 \pm 6.6$ - $90.8 \pm 0.5$ ; 12 hours -  $72.3 \pm 7.0$ - $81.2 \pm 2.9$ ; 24 hours -  $51.7 \pm 6.7$ - $63.7 \pm 3.9$ ,

Fig 3.12: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hour - early apoptotic -  $4.3 \pm 0.2$ - $9.5 \pm 3.6$ , late apoptotic/necrotic -  $4.3 \pm 0.4$ - $8.1 \pm 2.9$ , total dying -  $8.6 \pm 0.5$ - $16.8 \pm 6.9$ ; 12 hour - early apoptotic -  $6.6 \pm 0.5$ - $12.7 \pm 3.6$ , late apoptotic/necrotic -  $11.6 \pm 1.7$ - $16.8 \pm 4.8$ , total dying -  $18.3 \pm 1.6$ - $27.1 \pm 7.1$ ; 24 hour - early apoptotic -  $11.9 \pm 1.5$ - $16.8 \pm 2.8$ , late apoptotic/necrotic -  $23.2 \pm 3.1$ - $30.2 \pm 4.2$ , total dying -  $35.6 \pm 4.0$ - $47.0 \pm 6.7$ .

Fig. 4.5: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours -  $78.8 \pm 13.2$ - $88.8 \pm 4.7$ ; 12 hours -  $75.6 \pm 4.8$ - $80.7 \pm 4.0$ ; 24 hours -  $48.6 \pm 6.5$ - $52.2 \pm 4.7$

Fig 4.6: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours - early apoptotic -  $5.0 \pm 1.7$ - $8.3 \pm 6.6$ , late apoptotic/necrotic -  $5.7 \pm 3.1$ - $12.2 \pm 8.3$ , total dying -  $10.7 \pm 2.4$ - $20.4 \pm 6.5$ ; 12 hours - early apoptotic -  $7.8 \pm 2.0$ - $11.1 \pm 2.7$ , late apoptotic/necrotic -  $10.6 \pm 1.3$ - $13.1 \pm 2.7$ , total dying -  $18.9 \pm 2.0$ - $23.9 \pm 2.8$ ; 24 hours - early apoptotic -  $9.6 \pm 1.3$ - $11.5 \pm 3.2$ , late apoptotic/necrotic -  $36.9 \pm 3.0$ - $41.2 \pm 5.8$ , total dying -  $47.3 \pm 2.5$ - $50.8 \pm 3.2$ .

Fig. 4.7: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours – 78.6 $\pm$ 8.9-85.0 $\pm$ 3.4; 12 hours – 60.9 $\pm$ 5.6-68.7 $\pm$ 5.7; 24 hours – 29.1 $\pm$ 3.8-32.6 $\pm$ 7.0.

Fig 4.8: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours - early apoptotic – 8.1 $\pm$ 2.4-12.0 $\pm$ 6.1, late apoptotic/necrotic – 6.6 $\pm$ 1.1-10.0 $\pm$ 5.8, total dying – 14.7 $\pm$ 1.7-21.0 $\pm$ 4.0; 12 hours - early apoptotic – 12.7 $\pm$ 3.3-13.2 $\pm$ 2.7, late apoptotic/necrotic – 18.4 $\pm$ 3.2-24.9 $\pm$ 4.2, total dying – 31.0 $\pm$ 2.9-38.6 $\pm$ 2.5; 24 hours - early apoptotic – 16.2 $\pm$ 5.0-19.6 $\pm$ 2.7, late apoptotic/necrotic – 47.3 $\pm$ 4.6-52.7 $\pm$ 5.3, total dying – 66.0 $\pm$ 3.2-69.1 $\pm$ 2.4.

Fig. 4.9: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours – 78.8 $\pm$ 13.2-88.8 $\pm$ 4.7; 12 hours – 75.6 $\pm$ 4.8-80.7 $\pm$ 4.0; 24 hours – 48.6 $\pm$ 6.5-52.2 $\pm$ 4.7

Fig 4.10: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours - early apoptotic – 5.0 $\pm$ 1.7-8.3 $\pm$ 6.6, late apoptotic/necrotic – 5.7 $\pm$ 3.1-12.2 $\pm$ 8.3, total dying – 10.7 $\pm$ 2.4-20.4 $\pm$ 6.5; 12 hours - early apoptotic – 7.8 $\pm$ 2.0-11.1 $\pm$ 2.7, late apoptotic/necrotic – 10.6 $\pm$ 1.3-13.1 $\pm$ 2.7, total dying – 18.9 $\pm$ 2.0-23.9 $\pm$ 2.8; 24 hours - early apoptotic – 9.6 $\pm$ 1.3-11.5 $\pm$ 3.2, late apoptotic/necrotic – 36.9 $\pm$ 3.0-41.2 $\pm$ 5.8, total dying – 47.3 $\pm$ 2.5-50.8 $\pm$ 3.2.

Fig. 4.11: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours – 78.6 $\pm$ 8.9-85.0 $\pm$ 3.4; 12 hours – 60.9 $\pm$ 5.6-68.7 $\pm$ 5.7; 24 hours – 29.1 $\pm$ 3.8-32.6 $\pm$ 7.0.

Fig 4.12: The following are the ranges of the media baselines over treatment groups that were subtracted from the stimulated values: 4 hours - early apoptotic – 8.1 $\pm$ 2.4-12.0 $\pm$ 6.1, late apoptotic/necrotic – 6.6 $\pm$ 1.1-10.0 $\pm$ 5.8, total dying – 14.7 $\pm$ 1.7-21.0 $\pm$ 4.0; 12 hours - early apoptotic – 12.7 $\pm$ 3.3-13.2 $\pm$ 2.7, late apoptotic/necrotic – 18.4 $\pm$ 3.2-24.9 $\pm$ 4.2, total dying – 31.0 $\pm$ 2.9-38.6 $\pm$ 2.5; 24 hours - early apoptotic – 16.2 $\pm$ 5.0-19.6 $\pm$ 2.7, late apoptotic/necrotic – 47.3 $\pm$ 4.6-52.7 $\pm$ 5.3, total dying – 66.0 $\pm$ 3.2-69.1 $\pm$ 2.4.

## **Vita**

### **Kelcey M. Becker**

Kelcey Becker was born in Kansas City, Missouri in 1973. She grew up in Dallas, TX where she attended Richardson High School. After graduating high school in 1991, she moved to Williamsburg, VA and started her undergraduate studies at the College of William and Mary. Upon graduating in 1995 with a B.S. in Biology, she began working for Covance Labs in Vienna, VA. She worked for 2 years in the necropsy and histology labs at Covance. In 1997 she enter the MS program in the Dept. of Biomedical Sciences and Pathobiology at the Virginia-Maryland College of Veterinary Medicine.