

**EFFECTS OF LONG-TERM EXPOSURE OF NORMAL
C57BL/6J INBRED MICE TO 17 β -ESTRADIOL
ON GENE EXPRESSION IN LYMPHOCYTES:
mRNA ANALYSIS OF LYMPHOKINES AND *bcl-2/fas***

by
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Thesis submitted to the faculty of the Virginia-Maryland Regional College
of Veterinary Medicine, Virginia Polytechnic Institute
and State University in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

in

Veterinary Medical Sciences

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August, 1997

Blacksburg, Virginia

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(ABSTRACT)

It is now clear that human and animal exposure to estrogenic compound occurs through several sources. This include: i) naturally occurring endogenous estrogens, ii) exogenous or intentional estrogens for prophylactic (e.g. oral contraceptive) and therapeutic (e.g. as replacement therapy for ovulation in nulliparous women and in menopausal women, and in some men suffering from prostate cancer) purposes, iii) accidental via estrogenic chemical exposure (e.g. pesticides, industrial byproducts) and phytoestrogens (e.g. soybeans). It has long been recognized that estrogen, a female sex hormone, functions not only on the reproductive system, but also on various other systems including the immune system. Estrogens are thought to be of both physiologic and pathologic importance. Female in general, have better immune capabilities than males, a phenomenon attributed to the action of sex hormones on the immune system. There is also a female-gender bias in susceptibility to autoimmune diseases. Estrogens have been linked either directly or indirectly to the etiology and pathogenesis of various female-predominant autoimmune diseases. Estrogens have also been linked to the onset of cancer, and conditions where the immune system often malfunctions. Estrogen affects the functions of both B and T cells, possibly by regulating such factors as lymphokine gene expression and/or cellular death by apoptosis. However, the functioning of both B and T cells under the influence of long-term exposure to estrogen has not been fully understood.

The primary aim of this thesis was to investigate the effect of long-term exposure to 17 β -estradiol on lymphokine and *bcl-2/fas* (proto-oncogenes) mRNA expression. We evaluated the effects of estrogen on the expression of genes for lymphokines, which are essential for the

immune response. It is hypothesized that estrogen may regulate the immune system by modifying the expression of lymphokine genes and/or genes that regulate apoptosis.

The results demonstrated that long-term 17β -estradiol exposure reduced the viability of lymphocytes when compared to lymphocytes from placebo-treated mice. IL-2 and IFN- γ mRNA was consistently higher in ConA-stimulated lymphocytes from estrogen-treated mice ($P < 0.05$). The mRNA for TGF- β_1 lymphokine was also increased but was not consistent at all time points of incubation. The expression of IL-4 mRNA was not noticeably affected by estrogen treatment of mice. Long-term exposure to 17β -estradiol appear to have some influence on the mRNA expression of proto-oncogenes *fas* and *bcl-2* in splenic and thymic T lymphocytes. There was a trend of increased *bcl-2* mRNA expression in estrogen-treated mice compared to placebo-treated mice, whereas the mRNA expression of *fas* gene appeared to be lower compared to controls. Overall, these findings suggest that 17β -estradiol may selectively influence lymphokine and proto-oncogene mRNA expression. These results suggest that the one mode of modulation of the immune response by 17β -estradiol may be through alterations in the lymphokine and proto-oncogene expression.

Since estrogen-treatment markedly induces atrophy of the thymus and diminishes the cellularity of the lymphoid organs (e.g. Spleen), it became necessary to perform multiple assays on the same cells, particularly lymphokine and apoptosis gene expression. A secondary objective of this thesis was to investigate whether lymphocytes, which have undergone proliferation in Lympho-Pro™ assay (Alamar Blue assay), could be utilized for further analysis. In this regard, we found that a non-radioactive assay that utilizes Alamar Blue had significant advantages over the conventional ^3H -thymidine incorporation assay. By using cells from estrogen and placebo-treated mice in the Alamar Blue assay, we found that this assay not only allowed determination of lymphocyte proliferation, but also the assessment of mRNA expression, cytogenetics, apoptosis and immunophenotyping of the same lymphocytes.

Dedication

**I would like to dedicate this work to my parents:
Dongliang Yin and Xirong Wang,
as well as my husband Shi James Li,
and my daughter Yuanjing Li.**

Acknowledgments

I would like to express my utmost gratitude to my major advisor, Dr. N. Sriranganathan, for the valuable direction in molecular biology, professional guidance in experimental technology, and his tremendous support for all of my work. His selfless dedications of his time and patience are greatly appreciated. I would like to express thanks to Dr. S. Ansar Ahmed for the professional guidance with his solid knowledge in immunology, and autoimmune diseases, as well as his efforts in assisting me with the completion of my research project. I would like to thank to Dr. M. Crisman, who offered the helpful advice as my committee member. Without their directions and suggestions, I would not have been able to complete this work.

Thanks are extended to Dr. G. Schurig, Dr. M. Nagarkatti, Dr. V. Maxwell, Dr. P. Nagarkatti, and Dr. K. Elgert. They and Dr. S. Ansar Ahmed taught me contemporary knowledge of immunology in my topic in immunology class, which will play determinative role in my future career.

I would specially thank Mr. Todd Vaught, for his wonderful help in experimental skills and his knowledgeable support on using “Sigma Plot”. Thanks to Dr. Bruce Hissong, Mr. Paul G. Nordyke, and Dr. S. Arastu. I am very lucky to work with this group of people. Their help made my research progress smoothly.

My thanks go to Dr. Ramesh Vemulapalli for his knowledgeable direction about molecular biology.

I would like to thank Ms. Joan Kalintsky for Flow cytometry work, Dr. Chang-Guo Yang for statistical consulting, Mr. Terry Lawrence for media/graphics work, Mary Nickle and Cathryn A. Smith for excellent care of animals.

I appreciate Dr. Jean Whichard for her patience in correcting my thesis, thanks to Hamza Mohamed Eid, Tracy H. Vemulapalli, Lisa Tedora, Jeremy Boone, and Dr. Ruth Zhang. We all work in Dr. N. Sriranganathan’s lab. It is a friendly, nice, helpful environment for learning and working.

I also would like to thank Dr. John Lee, Dr. N. Sriranganathan for finance support, Dr. S. Ansar Ahmed and Dr. N. Sriranganathan for research grants support.

Finally, I would like to give my heartfelt thanks to my parents, Dong-Liang Yin, Xi-Rong Wang for their hope, education and foster, as well as their support and encouragement from overseas. Thanks also to my sister, brother-in-law, brothers and sister-in-law, Joan Q. Elangovan, A. R. Elangovan, Zhiqiang Yin, Zhiguo Yin, and Hui Chen. I would like to express the depth of my thanks and love for my husband Shi James Li, for his willingness, and sacrificing of his time and energy to support my study through the past three years. Special thank to my lovely daughter Yuanjing Li, for her funny letters and humorous children stories which brought me laughter and tears.

LIST OF ABBREVIATIONS

7-AAD	7-aminoacinomycin
AB	Alamar Blue
Ag	Antigen
BCG	Bacillus calmette-guerin
BrdU	5-bromodeoxyuridine
BSA	Bovine serum albumin
CAEV	Caprine arthritis-encephalitis virus
CD	Cluster differentiation
cDNA	Complement DNA
CL	Cardiolipin
CMI	Cell-mediated immune response
CNS	Central nervous system
Con-A	Concanavalin-A
DEPC	Diethyl pyrocarbonate
DEX	Dexamethasone
DHT	5 α -dihydrotestosterone
DP	Double positive
ds DNA	double strand deoxyribonucleic acid
E1	Estrone
E2	17 β -Estradiol
E3	Estriol
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IL	Interleukine
ITP	Idiopathic thrombocytopenic purpura
LT	Lymphotoxin
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAG	p-nitrophenol-N-acetyl-b-D-glucosaminide
NGF	Nerve growth factor
PC	Phosphatidylcholine
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PI	Propidium iodide
PI	Phosphatidylinositol
PMA	Phorbol myristate acetate
PO	Placebo
PPD	Purified protein derivative
PS	Phosphatidylserine
SS	Sjogren's syndrome
RT-PCR	Reverse transcriptase-polymerase chain reaction
RZ	Resazurin
SLE	Systemic lupus erythematosus
SRE	Steroid responsive-elements
TCGF	T cell growth factor
TCR	T cell receptor
TNF- β	Tumor necrosis factor- β
XTT	Sodium 3'-1-phenylamino-carbonyl(-3,4-tetrazolium)-bis (4-methoxy-6-nitro)benzene-sulfonic acid hydrate

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CHAPTER 1

LITERATURE REVIEW

Introduction

Traditionally, the immune system has been considered a relatively independent system providing protection against infection in human beings and animals. However, in the past several decades, we have learned that the immune system helps maintain homeostasis by interacting with other systems including the nervous and endocrine systems. The immune system, in turn, can be modulated by both the nervous and endocrine systems. Consequently, a novel interdisciplinary termed neuroimmunoendocrinology is gradually developing (Talal et al., 1987, Wilder, 1995). Alternatively, the immune system does not always play a protective role. Under certain circumstances, it plays a negative or an inadequate role affecting biological, biophysical, and biochemical metabolism in the body. The negative role of immune system may result in autoimmune diseases, and allergic disorders, while an inadequate role of immune system may result in immunodeficiencies.

Estrogen, a sex hormone, is important for the development of secondary sex characteristics in females. It affects various cells and tissues via the estrogen receptors in the cytoplasm and the nucleus. Estrogen is commonly used as a component of oral contraceptive, as replacement therapy for ovulation induction in nulliparous women and in menopausal women, and as therapy for a subset of men suffering from prostate cancer. It is now becoming apparent that women on estrogen therapy are increasingly prone to develop cancer and/or autoimmunity.

There are well-documented differences between males and females in terms of immune responses, both in clinical and experimental situations. Females respond better to immunization than do their male counterparts, as evidenced by their higher immunoglobulin production. Females have a greater ability to resist infections (Edinger, et al., 1972, Ansar Ahmed et al., 1985a, Olsen et al., 1996). In addition, females also have a better cell-mediated immunity as demonstrated by faster allograft rejection than that seen in males (Ansar Ahmed et al., 1985a, 1990). These differences between males and females disappear after castration of the male. On the other hand, females are more susceptible to most autoimmune diseases, including autoimmune thyroid diseases, systemic lupus erythematosus, rheumatoid arthritis, autoimmune diabetes mellitus and multiple sclerosis. Not only is the incidence of such diseases is higher in females, but the symptoms of autoimmune diseases in females can be severe. In experimental autoimmune diseases, testosterone and other male hormone have been shown to ameliorate these diseases (Ansar Ahmed et al., 1988, Lahita, 1993).

In recent years, many researchers have focused on the mechanism of estrogen modulation of immune responses. How does estrogen promote various autoimmune diseases? It has been documented that estrogen receptors are expressed not only in the reproductive system, but also in the immune system, both in lymphoid as well as stromal cells. In addition, administration of estrogen decreases the double-positive thymic cells and CD8+ cells. The ratio of CD4+/CD8+ is increased and cytotoxic cellular activity is reduced. Estrogen also promotes B cell function. Following administration of estrogen, autoantibodies can be induced, that are directed against

dsDNA, cardiolipin, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (Ansar Ahmed and Verthelyi, 1993a,b, Verthelyi and Ansar Ahmed, 1994). Cytokine production has been shown to be modulated by estrogen. Short-term estrogen-treated spleen cells incubated with Con-A produce higher levels of cytokines IL-2 and IFN- γ (Ansar Ahmed et al., 1985, Viselli et al., 1995). TNF- $\alpha\beta$ expression shows a dose-dependent biphasic profiles in T cell clones exposed to estrogen (Gilmore et al., 1997). Where as the level of IL-4 expression is not influenced by estrogen treatment (Dayan et al., 1997). Androgen administration enhances TGF- β 1 mRNA expression in the thymus and lacrimal glands, but suppresses it in the prostate glands (Kyprianou, et al., 1989, Huang, et al., 1995). However, it is not clear precisely how estrogen modulates and regulates T cell response.

In this study, we investigated the effects of long-term exposure of inbred mice to 17 β -estradiol on lymphokine and proto-oncogene mRNA expression of lymphocytes. In this study, orchietomized C57BL/6J male mice were used. The cytokines IL-2, IFN- γ , TGF- β 1, and IL-4 mRNA expression were investigated as representative T_H1 and T_H2 subtypes, respectively. Messenger RNA for *fas* and *bcl-2* proto-oncogenes was investigated. Both of these genes influence programmed cell death (apoptosis). Fas antigen blocks cell growth and induces apoptosis, whereas Bcl-2 enhances cell survival due to inhibition of apoptosis.

In this study, we extended the previous studies on the Alamar Blue assay (Lympho-Pro™), a non-radioactive colorimetric method used to indirectly measure lymphocyte proliferation (Ansar Ahmed et al., 1994). We tested, whether further analysis of lymphocytes is possible after determining proliferation and removing the indicator dye. Further, we also tested whether the biological activity of lymphocytes was altered after exposure to the Alamar Blue dye.

General review of T cells

T lymphocytes originate from hemopoietic stem cells in the bone marrow. These pre-T lymphocytes do not express T cell receptor (TCR) or accessory molecules, and do not have the ability to recognize antigen and therefore can not act as effector cells (Abbas et al., 1994). They migrate from the bone marrow to the thymus, where they undergo TCR-gene rearrangement, differentiation and selection.

Most of pre-T lymphocytes, in the cortex of the thymic microenvironment are associated with stromal cells, including interdigitating dendritic cells, epithelial cells and macrophages. These cells interact with pre-T lymphocytes by dendrites or long interconnecting cytoplasmic processes. Thymic hormonal factors, such as α 1-thymosin, β 4-thymosin, thymopoietin and thymulin, which are released from stromal cells in the thymic cortex contribute to T lymphocytes maturation. TCR and CD3 molecules are expressed early during T-cell maturation. TCR consists of either $\alpha\beta$ or $\gamma\delta$ heterodimers. The CD3 molecule is a complex of five amino acid chains that form three dimers: a heterodimer of gamma and epsilon chains ($\gamma\epsilon$), a heterodimer of delta and epsilon chain ($\delta\epsilon$), and a homodimer of two zeta chains ($\zeta\zeta$) or a heterodimer of zeta and eta chains ($\zeta\eta$). Both TCR and CD3 molecules associated together comprise the TCR/CD3 complex. TCR binds antigens directly, while CD3 molecules are required for expression of the TCR and for signal transduction. Any mutation in either the TCR or CD3 gene will lead to loss of expression of the entire TCR complex on the surface of the cell membrane (Abbas et al., 1994).

During differentiation in the thymus, the TCR repertoire accomplishes both positive and negative selection processes (Blackman, et al., 1990). Developing thymocytes with TCR's which are unable to recognize the self-major histocompatibility complex (MHC) molecules will be eliminated by programmed cell death (apoptosis). Consequently, two types of self MHC-restricted T cells remain: valid foreign antigen-specific and harmful self antigen-specific T cells (Abbas et al., 1994). During negative selection, any developing thymocytes that have high-affinity T cell receptors to autoantigens associated with self-MHC molecules or to self-MHC molecules alone are eliminated (Kuby, 1994, Takahashi et al., 1994). Through negative selection, potential self-reactive thymocytes will also be removed by programmed cell death. After TCR-gene rearrangement and positive/negative selection processes, mature T lymphocytes have a property that TCRs recognize non-self peptide antigens, which are associated with self-MHC molecules. Finally, mature T cells move to the medulla and leave the thymus into the circulation and peripheral lymphoid organs.

Following TCR/CD3 expression, the early thymocytes express CD4 and CD8 accessory molecules and differentiate into four groups of mature T-cell populations. These include double-negative CD3⁺ $\gamma\delta$ T cells; double-negative CD3⁺ $\alpha\beta$ T cells; single-positive CD4⁺ CD8⁻, CD3⁺ $\alpha\beta$ T cells; and CD4⁻ CD8⁺, CD3⁺ $\alpha\beta$ T cells. Single positive CD4⁺ and CD8⁺ T cell populations are the main functional cells. Most of thymocytes express low levels of TCR/CD3 complex. While mature thymocytes that are single positive CD4⁺8⁻ or CD4⁻8⁺ express high levels of CD3-TCR (Sentman et al., 1991). In addition to the expression of T cell receptor, accessory molecules CD3, CD4 and CD8, thymocytes also express CD28, CD45R, CD11aCD18, CD5 and CD2 as well as other molecules (Abbas,1994).

Mature T lymphocytes are divided into two different phenotypic subclasses based on distinctly expressed surface molecules--CD4⁺ or CD8⁺. Both molecules associate with TCR/CD3 complexes. The CD4⁺ subclass is restricted by MHC class II and mainly performs "helper" functions. They are termed T helper cells (T_H). The CD8⁺ subclass is restricted by MHC class I and functions as cytotoxic T cells (T_c) or suppressor T cells (T_s). T lymphocytes, as a major immune response cells, are involved in cell-mediated immune responses (CMI). Lymphokines secreted by T_H cells act as communication factors among T cells, B cells, macrophages, natural killer cells and others. T lymphocytes also regulate B cell-mediated immune responses by helping B cells to produce antibodies and enhancing macrophage activation. Cytotoxic T cells eliminate senescent cells, kill virus-infected cells and lyse tumor cells.

T helper cells (CD4⁺) are further divided into T_{H1}, T_{H2} and T_{H0} subsets based on the lymphokine patterns (Mosmann, 1996). T_{H1} cells synthesize IL-2, IFN- γ and lymphotoxin (LT). T_{H2} cells instead produce IL-4, IL-5, IL-6, IL-10 and IL-13. The lymphokine synthesis profile of T_{H0} cells overlap lymphokines which are produced by T_{H1} and T_{H2}. In addition all subsets are capable of secreting TGF- β (Mosmann et al., 1989, Gilmore et al., 1997). Regarding antibody production, T_{H1} cells assist in synthesis of more immunoglobulin G2a (IgG2a) isotype response than does the T_{H2} subset. The T_{H2} subset produces IgG1, IgA, and IgE antibody isotype response.

CD4⁺ cells regulate the balance between cell-mediated and humoral immunity by activation of functionally distinct T_{H1}, T_{H2} and T_{H0} cell subset. T_{H1} subsets proliferate optimally when

stimulated by macrophages or dendritic cells (Gajewski et al., 1989). It has been reported that T_H1 cells mediate delayed-type hypersensitivity (inflammatory) reactions, activate macrophages (Kakkanaiah et al., 1990) and perform a pro-inflammatory function. Low concentration of $IFN-\gamma$, produced by T_H1 cells, increase the immune responses (Reynolds et al., 1987). However some researchers reported immune suppression at high concentrations of $IFN-\gamma$. Lymphokine production by T_H2 cells triggers humoral immune mechanisms (Aust et al., 1996). Therefore, T_H1 subsets are called inflammatory T cells due to their role in inflammatory processes. T_H2 subsets are called T helper cells because of their assistance in humoral responses.

As $CD4^+$ T cells orchestrate the activities of B cells, macrophages, as well as CD8 cells (Janeway et al., 1994). T cell activation is a key step in initiating the immune response either by cell-mediated or humoral immune responses. There are two signals required to activate naive $CD4^+$ T cells. One of these signals is delivered by the association of a receptor on the T cell (TCR) with a peptide presented by MHC II molecule of an antigen presenting cell. The second signal is a costimulatory signal which is not antigen specific and does not initiate any immune response on the T cell, without TCR/CD3 liganded with Ag--MHC II. When T cell receptors are occupied by the peptide/MHC molecule and receives a positive costimulatory signal, the T cell will proliferate and differentiate into activated cells. However, T cells which receive the first signal without the subsequent costimulatory signal will undergo death (Webb et al., 1990) or become anergic (Kuby, 1994). Once the negative costimulatory signal is transferred into the cell, it may induce apoptosis (Boise et al., 1995).

The predominant costimulatory signal reported by numerous researchers are CD28 and CTLA-4 molecules presented on the surface of T cells and their ligands, B7-1 and B7-2, presented on the surface of the antigen presenting cells. The CD28 plays a positive costimulatory role. Whereas, CTLA-4 has dual roles, which is either a positive or a negative costimulatory role (Kearney et al., 1995; Boise et al., 1995).

Estrogen

Biological Characteristics

Basic structure: Estrogen, a steroid hormone, which possesses a basic structure of four carbon rings known as the cyclopentanoperhydro phenanthrene nucleus. All natural estrogens are C18 steroids which are derived from precursor C19 androgens by aromatization either in the gonads or in peripheral tissues (Hadley 1996).

There are three different structures of estrogen based on metabolic derivation: 17β -estradiol (E2), estrone (E1), and estriol (E3). Among these E2 is a predominant estrogen synthesized by the gonads.

Synthesis and secretion: The ovary is the main organ for synthesis and secretion of estrogen in females. During follicular development, two types of cells surround the oocyte. They are granulosa and thecal cells. C-19 androgens produced by thecal cells are metabolized to estrogen via two pathways. In the first pathway, C-19 androgens are delivered to the granulosa cells, wherein C-19 androgens are aromatized to estrogen by aromatase. Estrogen produced by this way serves a local function. In the other pathway C-19 androgens are aromatized within thecal cells. Estrogen derived through this pathway enters the circulation. The direct product of androgen

aromatization is 17 β -estradiol (E2), which accounts for 90% of the estrogen in circulation (Masi 1995). E2 can then be oxidized to E1 in the liver; and E1 can be further hydrated to E3.

In the male, the testis is the principle source of androgens. Aromatization of androstenedione and testosterone within testis or peripheral tissue is the major source of estrogen.

Estrogen receptor: Estrogens affect target cells via specific receptors. There are two main classes of estrogen receptors, α and β . Most investigators agree that estrogen can cross plasmamembranes and bind to nucleoplasmic receptors although this mechanism is still not clear. This activated nucleoplasmic receptor is then translocated to the chromatin. The receptor-steroid complex acts as a transcriptional enhancer by binding to specific DNA sequences known as steroid responsive-elements (SRE) which are 13 to 15 base-pair long (Yamamoto, 1985; Beato, 1989; Ansar Ahmed et al., 1990, Sarvetnick et al., 1990). This transcriptional enhancer activity is regulated by co-activator or co-repressor, which are called co-effector protein. once the co-activator is activated, a specific DNA sequence is derepressed and transcription increases. Specific proteins coded by mRNA are synthesized.

Several researchers have demonstrated that estrogen receptors are expressed not only in reproductive tissues, but also in many other systems, including the immune system. For instance, estrogen receptors have been identified in thymic cortical cells, thymocytes (Morgan et al., 1984; Carbone et al., 1986; Kawashima et al., 1992) and thymic epithelial cells (Grossman et al., 1979; Haruki et al., 1983; Nilsson et al., 1986; Kawashima et al., 1991). In the spleen, the estrogen receptors are found in supporting connective tissues and in splenic lymphocytes (Ansar Ahmed et al., 1990). The estrogen receptors are also found in suppressor T cells (CD8--positive), peripheral blood mononuclear cells, macrophages, monocytes, and B lymphocyte precursors. Estrogen receptors are not found in CD4⁺ (helper/inducer) human lymphocytes (Danel et al., 1983; Cohen et al., 1983; Stimson, 1988; Danel et al., 1988; Ansar Ahmed et al., 1990; Smithson et al., 1995; Ben-Hur et al., 1995). It is likely that estrogen receptors in the lymphocytes are E2R beta (Gustafsson, 1997).

Physiological function: Estrogen receptors are distributed among various tissues and systems in humans and animals. Therefore, it is not surprising that estrogens exert complex physiological regulation. Estrogens elicit biological responses not only within the reproductive system, but also on the central nervous system (CNS), the neuroendocrine system and the immune system. Specific effects of estrogen include: angiotensinogen and specific globulin production in the liver; decreased plasma cholesterol formation, water and sodium balance, lipid metabolism and maintenance of mineral deposition in bones (Hadley 1996). Estrogen has both short and long-term effects that may increase life span.

Estrogen and autoimmune diseases

Estrogen plays an important role in coordinating physiological processes and maintaining homeostasis. Under certain specific conditions, it is also used as a therapeutic agent. Estrogen can be used as replacement therapy during menopause and for ovulation induction in nulliparous women, as well as in prostatic cancer. Estrogen is a component of oral contraceptives. However, it has long been noted by the investigators that sex hormones interact with both the nervous and immune systems, thus contributing directly or indirectly to the etiology and pathogenesis of various autoimmune diseases. Diseases caused and/or complicated by sex hormone include:

autoimmune thyroid diseases (Williams et al., 1962; Chopra et al., 1974; Reuber et al., 1976; Khangure et al., 1977; Ahmed et al., 1980; Okayasu et al., 1981; William et al., 1986); systemic lupus erythematosus (Inman, 1978; Talal, 1979; Roubinian et al., 1977; Talal, 1981; Lahita et al., 1981; Walker et al., 1994); rheumatoid arthritis (Hench, 1938; Hench et al., 1949; Wingrave, 1978; Masi et al., 1984; Spector, 1989; Homo-delarche et al., 1991; Masi et al., 1995); autoimmune diabetes mellitus (Dotta et al., 1989; Olsen et al., 1996; Hawkins et al., 1996), idiopathic thrombocytopenic purpura (ITP) (Ahn et al. 1983); Sjogren's syndrome (Whaley et al., 1980; Apelgren et al., 1996) and multiple sclerosis (Birner, 1945; Sweeney, 1955; Schapira et al., 1966; Poser & Poser, 1983; Birk & Rudick, 1986; Birk et al., 1990; Duquette & Girard, 1993). Furthermore, the immune responsiveness manifests a significant difference due to gender difference. It appears that females are more susceptible to various autoimmune diseases than are males. This is true in both human and experimental animal models (Griensven et al., 1997). Moreover, estrogen therapy has been linked to development of cancer in human. It is tempting to speculate that the modulation of the immune system by estrogen may contribute towards oncogenesis. This thought gains further credence by the findings that prenatal exposure to synthetic estrogen, diethylstilbestrol (DES), increases the risk for the development of both cancer of reproductive tissues and several autoimmune diseases.

Clinical and experimental observations:

In humans: The susceptibility ratio of females to males is high for the following diseases: 25-50:1 for thyroid diseases; 9:1 for systemic lupus erythematosus (SLE); 2-4:1 for rheumatoid arthritis; 9:1 for sjogren's syndrome; 2-3:1 for autoimmune adrenal disease; 3-4:1 for scleroderma; 5:1 for autoimmune diabetes mellitus (Ansar Ahmed et al., 1985b, Homo-delarche et al., 1991) and 2-2.5:1 for multiple sclerosis (MS) (Duquette et al., 1992).

In animals: Similar sex-related autoimmune diseases have been identified in several animal species. In thyroiditis, affecting rats the susceptibility ratio of females to males is 3:1. The ratio is 4:1 for neonatal thymectomized rats. In thyroiditis obese female chickens are affected mainly. Systemic lupus and rheumatoid arthritis appear earlier in female mice than in males, including NZB, NZB/NZW F1, NZB/DBA/2 F1, NZB×SWR, NZB/CBA/N F1, MRL/lpr and MRL/n, and C57BL/6-lpr strains (Ansar Ahmed et al., 1985b). In addition, female NZB/W mice affected by SLE generally die within the first year of life due to glomerulonephritis, while male mortality rates during the first years are 10% or less (Roubinian et al. 1977; 1978). In polyarthritis, the ratio of females to males is 6:1 in LEW/N rats and 3:1 for LEW/N × F344/N FL rats. Hemolytic anemia incidence in mice is higher for females. In dogs, autoimmune thrombocytopenia occurs at a ratio of 2:1 females to males (Ansar Ahmed et al., 1985b).

Estrogen modulates autoimmune diseases:

Numerous studies have documented estrogen's influence on the immune system. Patients suffering from SLE or Klinefelter's syndromes metabolize estrogen abnormally, which elevates the production of 16 β -hydroxylated estrogen metabolites (16 β -hydroxyestrone and estriol). These metabolites have strong estrogenic activity, they bind estrogen receptor to prolong the estrogen effect in the body (Lahita et al., 1979, 1981; Talal, 1979, Ansar Ahmed et al., 1985b; Ben-Chetrit et al., 1994).

Healthy women receiving estrogen therapy for ovulation induction for primary or secondary infertility, some times develop SLE within one to three months (Ben-Chetrit et al.,

1994). The course of the disease can fluctuate with pregnancy or menarche, since estrogen levels during this times are frequently altered. The course of some autoimmune diseases are exacerbated by higher levels of estrogen. This is seen in SLE, autoimmune thyroiditis (Jungers et al., 1982; Amino et al., 1977 a), Grave's disease (Amino et al., 1977 b), rheumatoid arthritis (Hench et al., 1949; Apeltgren et al., 1996), and Sjorgren's syndrome (Apeltgren et al., 1996).

In addition, sex hormone replacement therapy modulates the progress of autoimmune diseases. The course of the SLE was reported to be accelerated by administration of estrogen-containing oral contraceptives, while disease amelioration was observed following withdrawal of the contraceptives (Ansar Ahmed et al., 1985b). The mortality and onset of SLE in NZB/NZW F1 male and female mice was enhanced by the administration of the testosterone-blocking drug flutamide (Walker et al., 1994). In contrast, administration of the male hormone 5 α -dihydrotestosterone (DHT) to NZB/NZW female mice will delay the onset of murine lupus or reduce the clinical signs, even allowing a normal lifespan (Roubinian et al., 1978; Ansar Ahmed et al., 1990). Women with SLE have lower androgen levels than do age-matched healthy female controls (Lahita et al., 1987, Apeltgren et al., 1996). In thymectomized-irradiated rat model of thyroiditis, the onset and severity of the diseases is markedly modulated by sex hormones (Ansar Ahmed et al., 1980). For example, testosterone was beneficial in both prevention (Ansar Ahmed et al., 1983) and curing diseases (Ansar Ahmed et al., 1986). In thyroiditis C3H mice, the production of autoantibodies to thyroglobulin was significantly higher in prepubertally orchietomized males. Autoantibodies against thyroglobulin were also higher for males treated with estrogen compared to sham-treated controls (Okayasu et al., 1981). In a model using an obese strain of chickens, autoimmune thyroiditis was prevented by administration of testosterone (Fassler et al. 1988). In Sjorgren's syndrome, the autoimmune infiltration of the lacrimal and salivary glands in MRL/Mp-lpr/lpr and NZB/NZW F1 mice can be significantly reduced by administration of testosterone (Ariga et al., 1989; Sullivan 1997).

Estrogen is not always pathogenic in autoimmune diseases. The clinical courses of some autoimmune diseases may be slightly ameliorated by estrogen treatment. Depending on dose, patient factors, and other treatment considerations (Ansar Ahmed et al., 1990; Carlsten et al., 1992, Apeltgren et al., 1996). Generally speaking, for the most part, however, excess estrogen tends to induce early onset and hasten progression of many antibody-dependent autoimmune diseases.

Effects of estrogen on T cells and cell-mediated immune response:

Estrogens demonstrate a strong thymolytic effect and affect several T lymphocyte functions, including T cell populations and cell-mediated immune responses. Thymic atrophy can be induced by castration or by administration of estrogen, and thymic cells (thymocytes) are the main affected component (Grossman, 1984). Several experiments have shown that treatment of rats with estradiol results in thymic involution. Histological examination demonstrated destruction of thymic lymphocytes, atrophy of thymic lobules, and an increase in thymic fat content (Grossman 1984). Under physiological condition, the lymphocytic count can be reduced to minimum at mid cycle, which coincides with peak of the serum estradiol concentration (Mathur et al., 1979; Danel et al., 1983). Further studies indicate that the estrogen targets double positive (DP) T cells during T cell differentiation and maturation, which causes this population to decrease. The ratio of CD4+ CD8-/CD4+ CD8+ subset of thymocyte populations was

significantly increased and the thymocyte was depleted after estrogen treatment for short-term in 3-4 week-old C57BL/6J mice (Screpanti et al., 1989. Silverstone et al., 1994). In earlier studies, estrogen reportedly increased mature thymocytes and reduced circulating CD8⁺ CD4⁻ T cells. It also caused a relative increase in T_H cells and a decrease in T_S cells analyzed by flow cytometry (Novotny et al., 1983; Ansar Ahmed et al., 1985a). Furthermore, the high ratio of CD4⁺ to CD8⁺ T cell population produced by estrogen or castration was reversed following administration of androgen (Olsen et al., 1991). A number of animal models have demonstrated that the total peripheral T cell activity was modulated by administration of estrogen *in vivo*. This is demonstrated by a shorter rejection time for graft in female estrogen-treated mice. This phenomenon resulted from either enhancement of T_H & inducer cell population or reduction in T_S & T_C cell activity (Olsen et al., 1996). Regardless of cell population changes, the end result is an increase in B cell activation, and higher immunoglobulin production (Grossman 1984). Other studies support that estrogen therapy in mice increases mature helper T cells (Novotny et al., 1983) and depletes suppressor/cytotoxic T cells (Ansar Ahmed et al., 1985b). On the other hand, in women suffering from estrogen deficiency, demonstrate a significant increase in absolute lymphocyte count and in percentage CD8⁺ cells. The ratio of CD4⁺/CD8⁺ is significantly decreased when compared to normal controls (Ho et al., 1991). Additionally, ovariectomized animals treated with estrogen were more susceptible to tumors (Ansar Ahmed et al., 1985b).

Effects of estrogen on lymphokine expression:

In recent years, several reports have addressed the effects of sex hormones (estrogens, progesterone and testosterone) on cytokine expression. The reported effect of estrogens on cytokine expression is variable. This may be due to different experimental systems, different methods for inducing autoimmune diseases, age of experimental animals, gender, tissues tested, and dose of estrogen used, as well as individual differences. A few reports showed that estrogens influenced the cytokine production. In human MS patients treated with 17 β -estradiol increased the production of both IFN- γ and IL-10, with different dose dependent profiles. Estrogen had a biphasic effect on TNF production. TNF was increased at low dosages of estradiol, and decreased at high dosages. Estradiol had no effect on the secretion of either IL-4 or TGF- β from T cell clones (Gilmore et al., 1997). Fox et al (1991) found that estrogen regulates IFN- γ production by increasing promoter activity. In this study, un-separated spleen cells from CD-1 male were incubated with estrogen. They found that short-term exposure to estradiol increased IFN- γ mRNA expression in response to Con-A stimulation in splenocytes. Viselli et al. (1995) studied the effects of androgen deficiency in male C57BL/6J mice. They found that IL-2 and IFN- γ cytokine production by Con-A stimulated un-separated splenocytes was higher in castrated males than in intact males. However, this finding has not been shown in anti-CD3-activated splenocytes from normal C3H mice (Araneo et al., 1991). IL-4 production from splenocytes was increased during pregnancy in normal mice (Dudley et al., 1993). In one experimental system, spleen cells were treated with Tamoxifen, an estrogen antagonist, or anti-estradiol antibody (16/6 Id). Several cytokines (IFN- γ , IL-2, TNF- α , IL-1 and IL-10) were reduced in both T_H1 and T_H2 cell types, while IL-4 was not affected (Dayan et al., 1997). Castration of normal mice leads to elevation of the production of IFN- γ in activated spleen cells (Viselli et al., 1995).

Programmed cell death in cell-mediated immune responses

Programmed cell death is a functional term for cell death that is required for proper development or function of an organ and system. PCD is a normal part of human and animal physiology. It occurs during cyclic or seasonal involution of gonads, epithelial and hematopoietic cell turnover, morphogenesis during embryonic development, and metamorphosis. Apoptosis is a term describing the morphological characteristics that occur in cells undergoing PCD (Cohen et al., 1991, 1992). A series of morphological features appear during apoptosis which distinguish it from traumatic or necrotic cell death. The main distinguishing features are, cell shrinkage, nuclear condensation, and formation of apoptotic bodies. Cells undergoing apoptosis lose 30% of their volume, while necrotic cells are characterized by swelling of all organelles and enlarge their volume. During apoptosis, DNA is degraded into multiple fragments. Examination of DNA through gel electrophoresis demonstrates a “ladder” pattern of bands. Other organelles relatively maintain or condensation. The cell remains intact or breaks up into membrane-bound apoptotic bodies (Duvall et al., 1986, Cohen et al., 1992, Wyllie, 1993, Krammer, 1993). The penultimate difference between necrosis and PCD is the necrotic cells loss of cell-membrane integrity. It leads to release of chemotactic cell contents, which elicits an inflammatory response to clear the debris.

In the immune system, there is evidence of apoptosis. As previously mentioned, pre-thymocytes migrate from the bone marrow to the thymic cortex, where they undergo differentiation and selection. Approximately 3% of these cells ultimately develop into mature T lymphocytes and are then distributed to peripheral circulation. In other words, 97% of them die in thymus. The mechanism of negative selection is accomplished by apoptosis. After positive selection, those cells, whose TCR recognizes self MHC complexes specific to foreign and self antigens remain. These remaining cells undergo negative selection, a process that eliminates the T cells whose TCRs bind with high affinity to self peptide antigens in association with self MHC molecules. Since this process deletes the self-reactive clonal T cells which represent a risk of autoreactivity, it assures self-tolerance. Conversely, breakdown of self-tolerance or damage to the negative selection process will result in autoimmunity and lead to autoimmune diseases.

During the immune response, high levels of growth factor (IL-2) and specific antigens are essential to maintain T-cell (CTL & T_H) growth and proliferation. Withdrawal of IL-2 or clearance of antigen will lead to apoptosis of T cells (Bishop et al., 1985, Cohen et al., 1985, Duke et al., 1986).

Death of CTL targets via apoptosis is observed *in vivo* and *in vitro* cell-mediated killing during viral infections, autoimmune reactions, and graft rejection (Matter, 1979, Russell et al., 1980, 1982, Duvall et al., 1986). It has been shown that DNA fragmentation in the target cell nuclei precedes ⁵¹Cr release. The experiment demonstrated that this process is mediated by a specific endonuclease and is inhibited by zinc ions, but not by inhibitors of protein synthesis. Results indicated that target cells exposed to CTL undergo internal disintegration rather than osmotic lysis (Russell, 1981, Duke et al., 1983). While freeze/thawing, heating, and lysing of cells with antibody and complement did not yield DNA fragmentation (Duke et al., 1983). In summary, apoptosis is important for T-cell maturation, maintenance of normal immune response, and balance of the immune response.

Cytokines and Proto-oncogenes

Cytokines are a group of low-molecular-weight regulatory proteins secreted by lymphocytes, mononuclear phagocytes, epithelial cells and fibroblasts in response to a number of antigenic challenge. Cytokines generally act as: regulators of growth and differentiation of various lymphocyte populations; initiators of the activation phase of T cell dependent immune responses; intercellular messenger molecules that are responsible for communication between immune cells and inflammatory system; and regulators of immune-mediated inflammation after binding to the receptors on the target cells.

Interleukin-2 (IL-2): IL-2, also called T cell growth factor (TCGF), is a small polypeptide mediator produced by the T_H1 subset of helper T cells, and in lesser quantities by CD8⁺ T cells. It is the main cytokine that induces progression of T cells from the G1 to the S phase of the cell cycle. IL-2 has both autocrine and paracrine function. It activates antigen-primed T_H and T_C cells, antigen specific T-cell clones, B cells, macrophages and NK cells in immune response. It stimulates T cells to proliferate, amplifies local immune responses, supports long-term growth and enhances immune cell reactivity. IL-2 also stimulates the growth of natural killer (NK) cells and enhances their cytolytic function. IL-2 acts on the B cells as a growth factor and a stimulus for antibody production.

Interleukin-4 (IL-4): IL-4 is a glycoprotein secreted by T_H2 subset of helper T cells. It acts as a growth factor for B lymphocytes. IL-4 stimulates B cell growth and B cell membrane receptor expression. It also induces class II MHC expression on a variety of cells. IL-4 turns on B cell production of IgG1 and IgE classes of immunoglobulins and promotes growth and differentiation of CTL. It also stimulates the growth of mast cells and facilitates a growth and differentiation factor of T cells, especially those of the T_H2 subset. IL-4 inhibits macrophage activation and in addition blocks most of the macrophage activating effects of IFN- γ (Paul, 1991).

Interferon- γ (IFN- γ): IFN- γ , a glycoprotein, produced by the T_H1 subset of T helper cells, T_C and NK cells (Billiau, 1996). IFN- γ targets a broad spectrum of cells, such as uninfected cells, macrophages, proliferating B cells, T_H2 cells and inflammatory cells. It generally augments cell-mediated immune response by up-regulating expression of class I and class II MHC molecules on target cells. Up-regulation of these MHC molecules enhances antigen presentation to differentiated effector lymphocytes. IFN- γ inhibits viral replication and increases the activation of macrophages to kill engulfed microbes. In addition, IFN- γ activates mononuclear and polymorphonuclear phagocytic cells; promotes the differentiation of T_H1 cells and maturation of CD8⁺ cells; and inhibits the proliferation of T_H2 cells. IFN- γ also blocks IL-4-induced class switch to IgE and IgG1.

Transforming growth factor- β (TGF- β): TGF- β is homodimeric molecule, which is defined as a regulator of neoplastic cell growth. It is secreted by platelets, macrophages and lymphocytes. The action of TGF- β are highly pleiotropic. It inhibits the growth of many cells, but stimulates the growth of others. TGF- β plays biphasic functions, which either inhibits or stimulates growth of the same cell type, depending upon the culture conditions and the presence of other growth factors. TGF- β is an important cytokine in inhibiting the proliferation of T cells and thymocytes, thus limiting inflammatory responses a promoting wound healing. It interferes with the activities of other cytokines such as IL-2.

Fas: The Fas antigen (also called APO-1 or CD95), a type I membrane-associated polypeptide, is a 45kd glycoprotein belonging to a family of receptors, which includes the tumor necrosis factor (TNF) and nerve growth factor (NGF). Fas is detectable in the thymus, heart, liver, ovary, kidney and lung (Watanabe-Fukunaga et al., 1992, Lacronique et al., 1996), and is expressed on mature T cells (single positive T cells). It is also expressed on double positive T cells and B cells (Drappa et al., 1993; Watanabe et al., 1995). The function of Fas antigen in mediation of apoptosis is through ligation with FasL, which is expressed on activated T cells (Kagi et al., 1996) or binding with Fas-antigen-specific monoclonal antibody. One of the most important roles of Fas mediated apoptosis in the immune system is the elimination of the autoreactive T cells in the thymus.

Bcl-2: *bcl-2* is a novel proto-oncogene. The protein is present in surviving mature thymocytes within thymic medulla. Most thymocytes in thymic cortex that have undergone apoptosis, do not have Bcl-2 (Hockenbery et al., 1990, Charles et al., 1991, Korsmeyer, 1992). Bcl-2 localizes on the inner mitochondrial membrane. It has been shown that Bcl-2 inhibits apoptosis in certain cell lines (Charles et al., 1991). Transgenic mice overexpressing Bcl-2 tend to develop tumors. This suggests that Bcl-2 is involved in cell survival mechanisms (Korsmeyer et al. 1992).

CHAPTER 2

SPECIFIC OBJECTIVES

I. Evaluation of Alamar Blue assay

The specific goals for Alamar Blue evaluation are to:

1. Compare the Alamar Blue assay with the ^3H -thymidine incorporation assay.
2. Determine the stability of color and fluorescence for the reduced form of Alamar Blue.
3. Determine the possible effects of Alamar Blue on lymphocyte biological activities.

II. Effects of long-term exposure of normal C57BL/6J inbred male mice to 17β -estradiol on gene expression in lymphocytes: mRNA analysis of lymphokines and *fas/bcl-2*

The specific goals for determining 17β -estradiol effects are to:

1. Determine whether or not estrogen affects the lymphokine mRNA expression;
2. Elucidate the specific effects of long-term exposure to 17β -estradiol on lymphokine mRNA expression, particularly IL-2, IL-4, IFN- γ , and TGF- β 1.
3. Determine the effects of long-term exposure to 17β -estradiol on proto-oncogene *fas* and *bcl-2* mRNA expression.

CHAPTER 3

A Dye-Based Lymphocyte Proliferation Assay that Permits Multiple Immunological Analyses: mRNA, Cytogenetic, Apoptosis, and Immunophenotyping Studies

A Dye-Based Lymphocyte Proliferation Assay that Permits Multiple Immunological Analyses: mRNA, Cytogenetic, Apoptosis, and Immunophenotyping Studies

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Abbreviations:

IL-2, Interleukin-2; IL-4, Interleukin-4; INF- γ , interferon gamma;
Con- A, Concanavalin A; 7-AAD, 7-aminoactinomycin D;
MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide;
XTT, (Sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-*bis*
(4-methoxy-6-nitro) benzene-sulfonic acid hydrate; BrdU,
5-bromodeoxyuridine; NAG, *p*- nitrophenol-N-acetyl-b-D-glucosaminide

ABSTRACT:

Alamar Blue and Resazurin, in the microenvironment of activated cells, undergo color change and also become fluorescent. By using the Alamar Blue dye, we have reported a non-radioactive colorimetric assay to indirectly determine proliferation of murine lymphocytes. We further show that the pattern of mitogen-induced proliferation assessed fluorometrically was comparable to the ^3H -thymidine incorporation assay (^3H -Tdr assay). Of practical importance is that the color/fluorescence changes were stable at 4°C in the dark for 3-4 weeks.

In immunological studies, it is important to further analyze lymphocytes that have undergone activation and/or proliferation. This is not possible with the standard ^3H -Tdr assay, which requires lysis of cells. In contrast, the Alamar Blue-based non-radioactive assay does not require cell lysis. We therefore tested the hypothesis that further analysis of lymphocytes is possible, after assessing the proliferation using Alamar Blue. Following assessment of proliferation in a 72-hour culture, the Alamar Blue dye was washed-off and cells were re-utilized to perform additional immunological analysis. Short-term exposure of lymphocytes to Alamar Blue was not detrimental to lymphocytes, as assessed by trypan blue exclusion and the Propidium Iodide (PI) assays. Exposure of dexamethasone-treated cells to Alamar Blue did not interfere with the performance of apoptosis assays, such as flow cytometric analysis of PI-stained cells and microscopic examination of ethidium bromide/acridine orange-stained cells. In addition, prior exposure of lymphocytes to Alamar Blue did not affect the analysis of chromosomal aberrations or the visualization of cell surface antigens by flow cytometry. Further, the expression of cytokine mRNA in lymphocytes previously exposed to Alamar Blue was similar to unexposed cells. Together, a notable advantage of this assay is that it now enables the investigator to maximize information by following or correlating proliferation with other immunologic events in the same cells.

Key words: *Alamar Blue, cell proliferation, mRNA, cytogenetics, apoptosis, Resazurin.*

INTRODUCTION

Mitogen-stimulated lymphocytes undergo alterations of membrane events and cell surface antigenic expression (e.g. acquisition of activation markers), replication of DNA (proliferation) and/or apoptosis (cell death). The mitogen-induced proliferation of lymphocytes is conventionally determined by ^3H -Tdr assay. The major advantage of this assay is its sensitivity. However, this assay also suffers from limitations including the concerns associated with the use of radioactivity, and the requirement of lysis of lymphocytes to determine the uptake of ^3H -thymidine. Therefore, intact cells are not available for further analysis.

In immunological research, the need to perform multiple analyses on the same cells is increasing. It may be necessary to determine not only proliferation of lymphocytes, but also to understand: (i) biochemical & molecular events in cells that have undergone proliferation; (ii) their surface antigenic characteristics; (iii) the induction/suppression of relevant genes and (iv) their susceptibility or resistance to cellular-death via apoptotic mechanisms. Furthermore, in studies which pertain to analysis of toxic effects of chemicals on lymphocytes, it may be necessary to relate proliferation with chromosomal aberrations. Maximizing the knowledge on same cells becomes especially relevant in situations with: (i) diminished numbers of cells (e.g. after cell sorting or from immunocompromised individuals) or (ii) shortage of animals.

Recently a non-radioactive colorimetric assay (also referred to as Lympho-Pro) was developed to indirectly determine mitogen-induced proliferation of lymphocytes (Ansar Ahmed et al. 1994). This technique employs a Alamar Blue dye, which in the presence of proliferating cells undergoes change in color from deep blue to shades of pink and the intensity of change to the pink color reflects the extent of proliferation (Ansar Ahmed et al., 1994; de Fries et al., 1995). The pattern of lymphocyte proliferation curve assessed colorimetrically was comparable to those obtained using the ^3H -Tdr assay. In this study we extended these findings to show that in the presence of stimulated lymphocytes, Alamar Blue dye not only changed color from blue to pink but also from a non-fluorescent to a fluorescent state. This enables assessment of proliferation by both colorimetric and fluorometric means. A significant advantage of this non-radioactive technique over ^3H -Tdr assay, is that it does not require the lysis of cells. In this study, we show that after determining proliferation with this technique, the dye can be washed off and cells reused to perform studies on immunophenotyping, chromosomal aberrations, apoptotic and lymphokine mRNA analysis. Thus, a major advantage of this dye-based assay is that it allows a researcher to perform further analysis on stimulated/proliferated cells.

MATERIALS AND METHODS:

Animals. Fifteen C57BL/6 and Five CFW (Swiss webster) adult male or female mice originally purchased from Charles River Laboratory were utilized in this study. These mice were fed on a commercial pelleted diet and maintained in our Center for Molecular Medicine & Infections Diseases (CMMID) laboratory animal facility (under 12 hours light/12 hours dark conditions), which is maintained in accordance with the animal welfare guidelines (IACUC).

Isolation of Lymphocytes. Spleen, thymus and lymph nodes were removed from mice under sterile conditions and gently teased on a metallic sieve (Sigma, St. Louis, MO) to obtain single cell suspension as previously described (Ansar Ahmed et al., 1987 and 1989). Erythrocytes in the cell pellet were eliminated by using Tris-ammonium chloride-lysis buffer (pH 7.2) at room temperature, followed by washing twice with fresh RPMI media (Mediatech, VA) as reported previously (Ansar Ahmed et al, 1989, 1994). The cell-suspensions were adjusted to 5×10^6 cells/ml in complete RPMI media supplemented with 10% of fetal bovine serum (Atlanta Biologicals. GA), 2 mM of L-glutamine (ICN Pharmaceuticals. CA) 1% non-essential amino acids (ICN), 50 IU/ ml of penicillin and 50 μ g/ ml of streptomycin (ICN).

Reproducibility of Experiments: All the experiments reported here were repeated at least three times (range 3 to 6 experiments).

Lymphocyte proliferation studies. One hundred microliters of splenic cell suspension (5×10^5 cells/well) were placed in 96-well sterile culture plates (Corning, NY) containing 100 μ l of complete RPMI media or various concentration of concanavalin A (Con-A, Sigma, MO) (0.1 μ g, 1.0 μ g, 10 μ g or 20 μ g/ml). The plates were incubated at 37°C/5% CO₂ under humidified conditions. In selected experiments, duplicate plates were set-up for concurrent determination of proliferation by ³H-Tdr and non-radioactive assays (Ansar Ahmed et al., 1994).

³H thymidine incorporation assay: This assay was performed according to procedures described in our previous studies (Ansar Ahmed et al., 1987, Ansar Ahmed and Sriranganathan, 1994, Ansar Ahmed et al., 1994). One microCurie of ³H thymidine was added to 5×10^5 cells/well during the last 24 hours of a 72 hour incubation. The cells were then harvested by filtration over glass fiber filter by a PHD harvester (Cambridge Technologies, MA) and the radioactivity uptake was determined by using a beta-counter (Beckman 8100 LS beta-counter Fullerton, CA). The data are expressed as Δ CPM [mean of CPM in mitogen-induced wells minus the mean of CPM in unstimulated wells], as described (Ansar Ahmed et al., 1994).

Non-radioactive (Lympho-Pro) assay. Alamar Blue™ (20 μ l/well; Accumed International Inc. Westlake, OH; or from Biosource Interanational/Tago Immunochemicals, Camars, CA) was added to stimulated and unstimulated lymphocytes after 48 hours of incubation and the plates were assayed 24 hours later (Ansar Ahmed et al. 1994).

Colorimetric Analysis: Absorbences were determined at wavelengths of 570 nm (reduced state) and 600 nm (oxidized state) using an optical density colorimeter plate reader

(Molecular Devices, Menlo Park, CA) 24 hours after the addition of Alamar Blue. It is recommended to subtract the absorbance at 600 nm from the 570 nm absorbance to achieve the specific absorbance (specific OD), since there is some overlap between absorbance at 570 nm and 600 nm (Ansar Ahmed et al. 1994).

Fluometric Analysis: The absorbance of cultures exposed to Alamar Blue was measured by CytoFluor™ II multiwell plate reader (PerSeptive Biosystems. Inc. MA) at wavelengths of 530 nm excitation, 590 nm emission, and a gain of 35.

In our previous studies, there was a high degree of correlation in the pattern of proliferation curves between the radioactive ³H-Tdr and Alamar Blue-based non-radioactive assay (Ansar Ahmed et al., 1994). Similar results were seen in our initial studies. Therefore, in all subsequent studies, Alamar Blue-based non-radioactive assay was exclusively employed. After the determination of proliferation, the cells were washed three times with RPMI-media and cells readjusted to appropriate concentrations in RPMI-media (supplemented with 10% fetal bovine sera) for the other assays reported below.

Studies on stability of Alamar Blue: In selected studies, following the assessment of proliferation, the plates were stored in the dark at 4°C. The plates were analyzed periodically for 3-4 weeks by both colorimeter and fluometer to evaluate the stability of the fluorescence or absorbance changes in Alamar Blue.

Cell viability assay: Splenocytes or thymocyte that were cultured with and without Alamar Blue during the last 24 hours of a 72-hours culture, were washed three times with media and the cell viability determined by the trypan blue exclusion method (Ansar Ahmed et al., 1987).

Assay for Apoptosis and Cell Cycle. One hundred microliters of thymocytes (5×10^6 cells/ml) were placed in a 96-well sterile culture plate containing 100 μ l of an apoptosis-inducer, dexamethasone (DEX) (0.1 μ g/ml or 1 μ g/ml) or 100 μ l of complete media alone and incubated for 6 hours at 37°C (5% CO₂) under humidified conditions. These conditions were shown to induce apoptosis in our preliminary studies. The cells were then washed with RPMI media and resuspended in 200 μ l of complete RPMI media to a cell concentration of 5×10^5 cells/well. Alamar Blue (20 μ l) was added to all wells and incubated for another 24 hours under the same conditions as above. Morphological assays for apoptosis using ethidium bromide-acridine orange staining and cell cycle analysis with PI staining were performed after the dye in culture was washed off (as mentioned previously) .

Ethidium Bromide and Acridine Orange Apoptosis Assay: Twenty five microliters of 5×10^6 cells/ml in complete RPMI media were gently mixed with 1 μ l of the dye mixture (100 μ g/ml ethidium bromide and 100 μ g/ml acridine orange) and examined under 40 \times magnification using a fluorescent microscope to visualize and count cells with aberrant chromatin organization. Acridine orange will stain cells undergoing apoptosis, while ethidium bromide identifies nonviable cells. Therefore, a mixture of acridine orange and ethidium bromide was employed in this experiment to identify viable, apoptotic and nonviable cells (Coligan et al.,

1994). This assay identifies four phases: (1) viable cells with normal nuclei (VN); (2) viable cells with apoptotic nuclei (VA); (3) non-viable cells with normal nuclei (NVN); (4) nonviable cells with apoptotic nuclei (NVA). The percentage of apoptotic index = $(VA + NVA) \div (VN + VA + NVN + NVA) \times 100$, the percentage of necrotic cells = $(NVN) \div (VN + VA + NVN + NVA) \times 100$, while the percentage of dead cells = $(NVN + NVA) \div (VN + VA + NVN + NVA) \times 100$.

Cell cycle analysis. Cells treated with the PI can be utilized to assess not only the stages of cell cycle (G_0/G_1 , S, G_2/M), but also to identify apoptotic cells (hypodiploid, sub G_0 peak). Half a million cells were pelleted at $1000 \times g$ for 5 minutes and the cells were mixed with 250 μ l of Vindelov's PI stain solution (1.21 g TRIS base, 584g NaCl, 10 mg RNase, 50.1 mg PI, 1 ml Nonidet P-40, pH 8.0) and 250 μ l of standard azide buffer (Robinson et al., 1993). Cells were incubated at 4°C overnight and analyzed using an Epics-XL flow cytometer (Coulter, FL).

Total RNA extraction. In order to avoid contamination with ribonuclease during extraction of RNA, all glassware were baked at 180°C for 8 hours, aerosol resistant filter tips were employed, all solutions were treated with 0.01% DEPC (diethyl pyrocarbonate) and latex gloves were worn during the entire procedure. Splenic cells (5×10^5 cells), both unexposed and exposed to Alamar Blue, were washed twice with fresh RPMI and transferred into autoclaved microcentrifuge tubes. TRIzol™ reagent (500 μ l: Life Technologies, MD) was added to each tube and the cells were homogenized and incubated for 10 minutes at room temperature. Chloroform (100 μ l) was then added to the sample and the samples were gently mixed and incubated at room temperature for 15 minutes followed by centrifugation at $12,000 \times g$, 4 °C for 15 minutes (MTX-150, Peninsula, CA). The aqueous phase (above the interphase) was transferred to a microcentrifuge tube and 250 μ l of isopropyl alcohol added to precipitate RNA. The mixture was incubated at room temperature for 10 minutes and centrifuged at $12,000 \times g$, 4 °C for 15 minutes. The RNA pellets were washed with 0.5 ml of 75% ethanol and pelleted again.

Determination of total RNA. The RNA pellets were dissolved in 500 μ l of DEPC-treated distilled water. The total RNA concentrations were determined by optical measurement at 260 nm using a GILFORD Response™ spectrophotometer (Ciba Corning Diagnostics Corp. Massachusetts) and using a conversion factor, 1 absorption unit₂₆₀ is = 35 μ g/ml.

Cytokine mRNA analysis. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect β -actin, IL-2, IL-4, IFN- γ , and TGF- β_1 mRNA expression (Oliveira et al., 1994). For the first strand synthesis of cDNA, total RNA from 5×10^5 cells was suspended in a 30 μ l reaction solution containing 0.75 μ g oligo(dT)₁₂₋₁₈ (GIBCO BRL, Gaithersburg, MD) 1.25 mM of dNTP, 15 units of RNasin (Promega Corporation Madison, WI), 3 μ g of acetylated BSA, 1 \times RT buffer, 3 μ l of 0.1M DTT and 1.5 μ l (300 units) of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The reaction mixture was incubated at 37°C for 1 hour. The PCR reactions were performed in a 50 μ l volume, containing 5 μ l of the cDNA samples from

the previous step and they were subjected to PCR amplification with 2.5 mM MgCl₂, 0.25 mM of dNTP, 1 × PCR buffer, 2µg of acetylated BSA, 1µM each specific primer, and 0.5µl (2.5 units) of Taq DNA polymerase (GIBCO, BRL). The PCR reactions were performed using a Ominigene TR3 CM110 thermocycler (Hybaid Limited, TW) under the following conditions: denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C and amplification for 1.5 minutes at 72°C, with an additional 10 minutes of extension at 72°C. Twelve microliters of PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide (0.45µg/ml) at 75 volts for analysis of cytokine mRNA expression. The RT-PCR products were quantified using a scanning densitometer (Molecular Dynamics, CA).

Flow cytometric analysis. Unstimulated and Con-A stimulated splenic cells (5×10^5 cells/ well) cultured with and without Alamar Blue, were washed three times with fresh RPMI 1640. Aliquots of cells were stained with fluorescein isothiocyanate (FITC) conjugated anti-mouse CD90.2 (Thy1.2), or its isotype anti-rat IgG_{2a}, κ control antibodies; FITC anti-mouse CD2, or its isotype FITC anti-rat IgG_{2b}, λ control antibodies (Pharmingen San Diego, CA) as previously described (Ansar Ahmed et al , 1989, 1994). These stained cells were analyzed using an Epics-XL flow cytometer and data presented as histograms (Ansar Ahmed and Sriranganathan, 1994).

Cytogenetic Studies. Lymph node cells (5×10^6 cells/ml) in complete RPMI media, in duplicate culture flasks, were stimulated with PMA (10 ng/ml) (Sigma, ST. Louis, MO), and ionomycin (0.5 µg/ml) (Sigma, MO) and incubated for 72 hours in a humidified 5% CO₂ atmosphere at 37°C. Alamar Blue (20 µl/5x10⁵ cells) was added in the last 24 hours of a 72-hour incubation. Cells without Alamar Blue were cultured for control data. Colchicine (Sigma, MO, 0.1 µg/ml) was added 2 hours prior to cell harvesting in order to arrest the cells at metaphase. Cells were washed twice in RPMI media, harvested, and slides prepared according to the standard method (Moorhead et al., 1960; Fatima (*nee* Arastu et al., 1995). The slides were air dried and stained with Giemsa (Sigma). Structural chromosomal aberrations (gaps, breaks, acentric fragments, exchanges) and numerical chromosomal aberrations (polyploids) were scored in 100 metaphase cells per/treatment/ animal.

Comparison of Alamar Blue and Resazurin studies: One hundred microliters of splenic or thymic lymphocyte suspension (5×10^6 cells/ml) were placed in 96-well sterile culture plates (Corning, NY) containing 100µl of complete RPMI media with or without Con-A (10µg/ml). The plates were incubated at 37°C (5% CO₂) under humidified condition for 48 hours. Alamar Blue or Resazurin (Sigma, St. Louis, MO) was added to cells at ten or twenty microliters volume. Filtration of Resazurin dye (by 0.45 µM filters) was found to be necessary to attain consistency in color/fluorescence changes. The fluorescent change were assayed 24 hours later by CytoFluor II.

Statistical analysis. A Spearman rho test was used to determine the coefficient of correlation between Alamar Blue and ³H-thymidine methods of determining proliferation of lymphocytes.

The Student's t-test and chi-square (χ^2) test, where appropriate, were employed to assess the significance of the data. The data are presented as mean \pm SEM.

RESULTS

Comparison Of Mitogen-induced Splenic Cell Proliferation by Non-Radioactive assay (Lympho-Pro) with ^3H -thymidine assay. The patterns of Con-A-induced proliferation curves determined colorimetrically, as absorbances, (Fig. 1a) or fluorometrically, as fluorescence units, (Fig. 1b) were similar to that noticed in the 'gold Standard' ^3H -thymidine incorporation assay (Fig. 1c). In all methods of analysis, 0.1 $\mu\text{g}/\text{ml}$ of Con-A induced minimal proliferation, 10 $\mu\text{g}/\text{ml}$ of Con A induced optimal proliferation, while 20 $\mu\text{g}/\text{ml}$ of Con A inhibited proliferation (possibly due to cellular death). This pattern was evident in both CFW (Swiss Webster) and C57Bl/6 mice. In all subsequent studies only C57BL/6 mice were used.

Stability of change in Alamar Blue: As seen in figure 2, the pattern of color changes (2a) and fluorescence changes (2b), which is indicative of cellular proliferation, was relatively unchanged for 3-4 weeks.

Exposure of Alamar Blue and Viability :We next investigated the possible toxic effects of exposure of lymphocytes to Alamar Blue added during the last 24 hours of a 72 hour culture. Prior exposure of cells to Alamar Blue did not affect the viability as assessed by Trypan Blue and PI exclusion assays. The percentage of viability of cells unexposed or exposed to Alamar Blue were 58% \pm 2.5 and 57% \pm 1.9, respectively (n = 8), P = 0.75. PI analysis also revealed similar cell viability patterns (Table Ia).

Exposure of cells to Alamar Blue does not affect apoptosis: Apoptosis of splenic lymphocytes or thymocytes was deliberately induced by exposure to dexamethasone (0.1 $\mu\text{g}/\text{ml}$, and 1.0 $\mu\text{g}/\text{ml}$) and cells subsequently incubated without or with Alamar Blue. Apoptosis in washed cells was determined by PI analysis. Table Ia shows prior exposure of splenic lymphocytes to Alamar Blue did not interfere with this apoptosis. Similar results were also obtained from dexamethasone-induced apoptosis of thymocytes (data not shown). Apoptosis was also evaluated microscopically in ethidium bromide/acridine orange stained cells. There was no significant difference in cell viability between cells unexposed and exposed to Alamar Blue. Table Ib shows the percentage of the apoptotic index, and necrotic and dead cells.

Alamar Blue exposure and Cell Cycle Analysis: Prior exposure of lymphocytes to Alamar Blue did not impede with the cell-cycle analysis (Table Ia).

Alamar Blue did not Interfere with total RNA synthesis or Expression of Cytokine mRNA:. The total RNA amount (mean \pm SEM) from cells grown in the media unexposed or exposed to Alamar Blue was 0.645 μg \pm 0.17, and 0.569 μg \pm 0.12, respectively. The total amount of RNA from Con-A (1.0 $\mu\text{g}/\text{well}$)-stimulated cells that were unexposed or exposed to

Alamar Blue was $2.393 \mu\text{g} \pm 0.68$ and $2.352 \mu\text{g/ml} \pm 0.78$, respectively ($p =$ not significant). Thus, Alamar Blue does not appear to interfere with RNA synthesis.

We further examined whether prior exposure of splenic lymphocytes to Alamar Blue interferes with the determination of specific cytokine mRNA expression by RT-PCR. The specific RT-PCR products for β -actin, IL-2, IL-4, IFN- γ , and TGF- β 1 cytokine mRNA were analyzed on a agarose gel-electrophoresis and quantitated by scanning densitometer. As depicted in Figure 3, the specific lymphokine mRNA expression was similar for each cytokine regardless of the exposure of lymphocytes to the Alamar Blue dye.

Effects of Alamar Blue on Expression of Cell Surface Antigens: It is often necessary to determine the phenotypic characteristics of cells that have been stimulated and/or have undergone proliferation. Following the determination of proliferation, the numbers of CD90.2 (Thy-1.2) and CD2 positive cells were identified by flow cytometry in order to determine whether or not Alamar Blue dye influences the expression of these molecules on cell surface. Splenic cells that were exposed to Alamar Blue have similar fluorescent intensity patterns and percentages of anti-CD90.2 or anti-CD2 positive cells (figure 4). These data suggest that Alamar Blue does not interfere with the binding of antibodies to the cell surface antigens, and also suggest that the expression of cell surface molecules are not affected by this dye.

Alamar Blue does not induce Chromosomal aberrations: The total structural chromosomal aberrations in lymphocytes exposed to Alamar Blue was 6.5%, compared to 5.75% in untreated lymphocytes (Table II; $p =$ not significant).

Comparison of Alamar Blue and Resazurin: Con-A stimulated splenic lymphocytes were exposed to various quantities of Alamar Blue or Resazurin. The pattern of cell proliferation determined by colorimetrically or flurometrically are shown in figure 5. The pattern of proliferation identified by Alamar Blue or by Resazurin were similar ($p > 0.05$; not significant)

DISCUSSION

Mitogen-induced lymphocyte proliferation assays are useful in assessing the functionality of T or B cells, and are therefore commonly employed in both research and clinical settings. The most common method employed to determine proliferation is the ^3H -Tdr assay. This is a highly sensitive and definitive assay which is based on the uptake of ^3H -thymidine into the DNA backbone of the cells undergoing division. However, there are several shortcomings to this assay, such as the use of radioactive nucleotides, potential health hazard to the user, waste generated is an environmental concern, it requires restricted space for radioactive use, it is labor intensive, and the need for specialized equipment. In order to obviate the use of radioactive reagents, a number of non-radioactive lymphocyte proliferation assays have been reported in recent years. These include: (1) the reduction of tetrazolium salts such as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Mosmann, 1983) and Sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT; Scudiero et al., 1988; Roehm, 1991); (2) incorporation of a pyrimidine analog,

5-bromodeoxyuridine (BrdU), (Raza et al., 1984; Porstmann, et al., 1985; Magaud et al., 1988; Huong et al., 1991); (3) turnover of lysosomal hexosaminidase, *p*- nitrophenol-N-acetyl-b-D-glucosaminide (NAG test) (Landegren, 1984), and (4) detection of proliferation-related antigen in human lymphocytes (Palutke et al., 1987) or in cell lines derived from the bladder cancer of rats (Hojo et al, 1992). The BrdU and NAG tests have been reported to be comparable to ³H-Tdr assay (Wemme et al., 1992). However, a number of these assays involve multiple steps which increase chance of error. The XTT assay is relatively simple and cleavage of XTT results in an aqueous formazon product. However, efficient bioreduction of XTT is dependent upon inclusion of electron coupling reagents such as menadione or phenazine methosulfate in the assay system (Roehm et al., 1991). More recently, we have reported a simple, essentially a one-step non-radioactive assay which employs Alamar Blue dye to indirectly assess proliferation of lymphocytes (Ansar Ahmed et al., 1994). This non-radioactive assay has also been adapted to assess mitogen-induced proliferation of peripheral blood lymphocytes from chickens (Ansar Ahmed, et al., 1996., Gogal et al., 1997) and in humans (de Fries et al., 1995). This non-radioactive assay has also been used to determine cellular proliferation of different cell lines in oligodeoxynucleotide research (Squatrito et al., 1995) and to assess cytotoxicity in several tumor cell lines (Page et al., 1993). Finally, this assay has also been used to test the minimum inhibitory concentrations of antimicrobial agents for *M tuberculosis* (Yajko et al., 1995), Yeast (Tiballi et al., 1995a), *S.cervisiae* (Tiballi et al., 1995b), and *Candida albicans* (Pfaller et al., 1994., Espinel-Ingroff et al., 1995) and as an indicator for microbial proliferation (Baker et al., 1994).

We find that the pattern of lymphocyte proliferation curves in response to various doses of Con-A, assessed colorimetrically or flurometrically, is comparable to the ³H-thymidine incorporation assay (see figure 1). Of practical importance is that the color and fluorescent changes are stable for 2-3 weeks at 4⁰C in the dark (figure 2). This will allow investigators to analyze plates at their convenience, although it should be noted that cells presumably are not viable after storage at 4⁰C. The addition of Alamar Blue to cell cultures for a period of 24 hours (this study) or for 48 hours (Ansar Ahmed et al., 1994) had no apparent toxic effects on lymphocytes, based on exclusion of vital dyes such as trypan blue or PI (Table I). This suggests that the integrity of cell membrane is not altered by 24-48 hour exposure to Alamar Blue at the concentration used.

In immunologic research, it is becoming increasingly necessary to follow and correlate multiple cellular events. Maximizing information is particularly useful in situations where only limited numbers of lymphocytes are available (for e.g. in animals treated with cytotoxic or immunosuppressive agents or in situations where only finite numbers of cells are available after cellular purification steps). It is thus advantageous to characterize the morphological and molecular changes in cells that have been activated to undergo proliferation. These analyses are not feasible using sensitive ³H-thymidine or BrdU assays, since these methods require disruption of cells to extract the DNA. The MTT assay also suffers from a similar shortcoming, in which solubilization of formazon crystals (a critical step for this assay) requires vigorous mixing of cells with acid-isopropanol or DMSO. In contrast, in the non-radioactive Lympho-Pro assay, cellular lysis is not required for assessing proliferation or viability. Conceivably, after the dye has been washed off, cells can be therefore reused for additional analysis. In this study we

sought to determine which assays are possible to perform after evaluating proliferation/viability. Flow cytometric analysis of activated and inactivated cells revealed that the percentages of positive cells and their fluorescent intensity patterns were similar, regardless of the exposure to Alamar Blue (figure 4). In our experience, FITC-conjugated reagents do not interfere with Alamar Blue. PE-conjugated reagents can also be used, provided that cells are thoroughly washed and the flow cytometer settings optimized (Ansar Ahmed et al, 1994). Flow cytometry is also useful in analyzing PI stained cells at different stages of cell cycle and previous exposure of cells to Alamar Blue did not interfere with the cell cycle analysis. Thus after assessing proliferation, the same cells can be used to analyze cell activation, expression of proliferation-related cell surface antigens or the stages of the cell cycle.

Exposure of lymphocytes to Alamar Blue did not interfere with the total RNA levels or specific cytokine mRNA synthesis (figure 3). This would now allow analysis of activation-induced genes (via specific mRNA) in cells that have undergone stimulation or proliferation. Analysis of cells undergoing cell death by apoptosis is of major interest in biomedicine. Apoptosis can be induced in cells by a variety of means including activation of cells. Apoptosis was deliberately induced in thymocytes and splenic lymphocytes by dexamethasone. We found that cells exposed to Alamar blue had the same number of apoptotic and viable cells as controls, suggesting that this dye does not interfere with these assays or induce apoptosis. However, another DNA-binding dye, 7-aminoactinomycin D (7-AAD), which is used to identify early and late apoptotic/necrotic cells (Schmidt et al., 1994) cannot be used with cells exposed to Alamar Blue. These two dyes appear to interfere in flow cytometric analysis (Ansar Ahmed and Vaught, Unpublished information). Alamar Blue did not increase the total number of chromosomal aberrations, suggesting that this dye does not provoke genetic damage (Table II). Thus mutagens, carcinogens and teratogens, which have deleterious effect on lymphocytes in terms of proliferation and chromosomal aberrations, can be evaluated by using this approach.

We recently became aware of another dye, Resazurin, which appears to have properties that are very similar to the Alamar Blue dye in lymphocyte proliferation assays (figure 5). In a limited series of experiments, we found that the pattern of Con-A-induced proliferation using Resazurin was comparable that of Alamar Blue. We are currently performing extensive studies to delineate mechanisms underlying the action of Alamar Blue and Resazurin. It was initially believed by the manufacturer of the Alamar Blue that cells undergoing proliferation release metabolites which effectively reduce these dyes to induce color/fluorescence changes. However, our ongoing studies suggest that this may not be entirely true, since addition of Alamar Blue to cell-free culture supernatants, derived from mitogen-stimulated cells or from actively-growing cell lines, did not change color/fluorescence. This suggests that the presence of activated cells is necessary for color/fluorescence changes. We also have evidence to show that “reduced” Alamar Blue dye binds or enters the cells (unpublished data).

In conclusion, exposure of cells to Alamar Blue does not appear to alter the cell membrane characteristics as assessed by the exclusion of vital dyes, apoptosis assays, and flow cytometric analysis of cell surface antigens. It also enables performing mRNA, cell cycle and cytogenetic studies. Thus, subsequent to assessing proliferation or viability of cells by this approach, multiple assays can be performed on the same cells to maximize information.

ACKNOWLEDGMENTS: This work is supported by NIH 1R01-ES08043-01 grant award and Lupus foundation of America grant awards to S.A.A. We thank Mrs. Mary Nickle for excellent care of animals, Mr. Terry Lawrence for graphics work and Ms. Joan Kalinsky for Flow cytometry work.

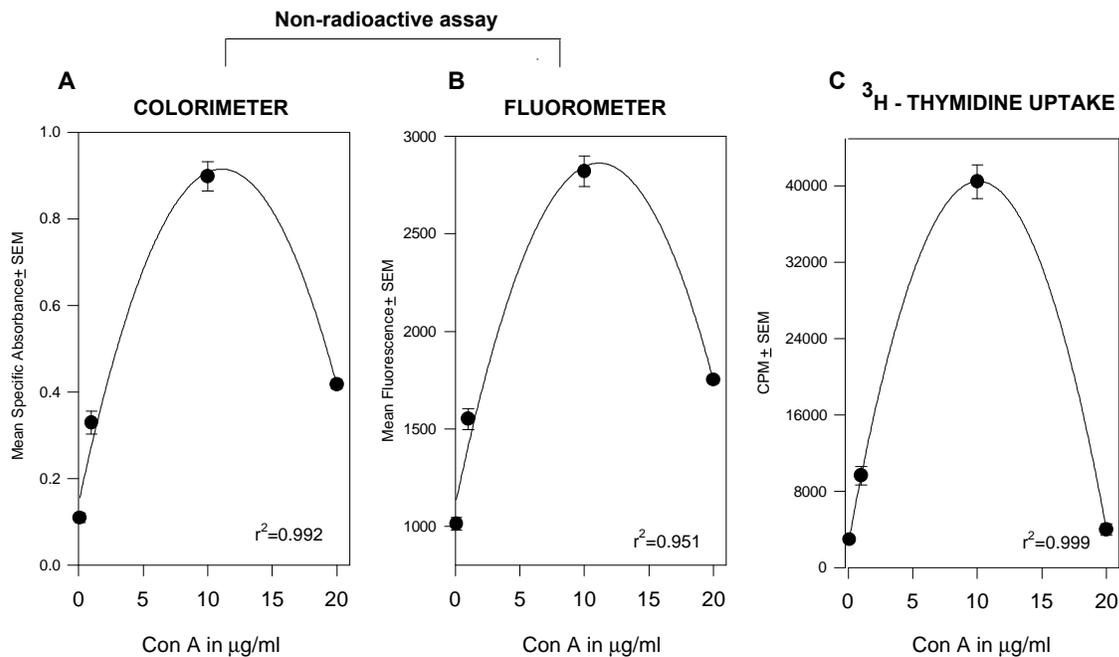


Fig.3.1: Mitogen-induced proliferation of lymphocytes was assessed by the Alamar Blue-based non-radioactive assay (Lympho-Pro) and by the ^3H -thymidine incorporation assay. Splenic lymphocytes were stimulated with various doses of Con-A and aliquots of cells were exposed to Alamar Blue in media during the last 24 hours of a 72 hour culture. Duplicate sets of plates were concurrently analyzed by the ^3H -thymidine assay. The lymphocyte proliferation was analyzed by (a) a colorimetric assay based on the color spectrum. The data is presented as specific absorbances (570 nm *minus* 600 nm); (b) a fluorometric based assay on the fluorescent spectrum (530 nm excitation/590 nm emission); and (c) [^3H] thymidine assay. This figure represents data from six separate experiments. (n = 6).

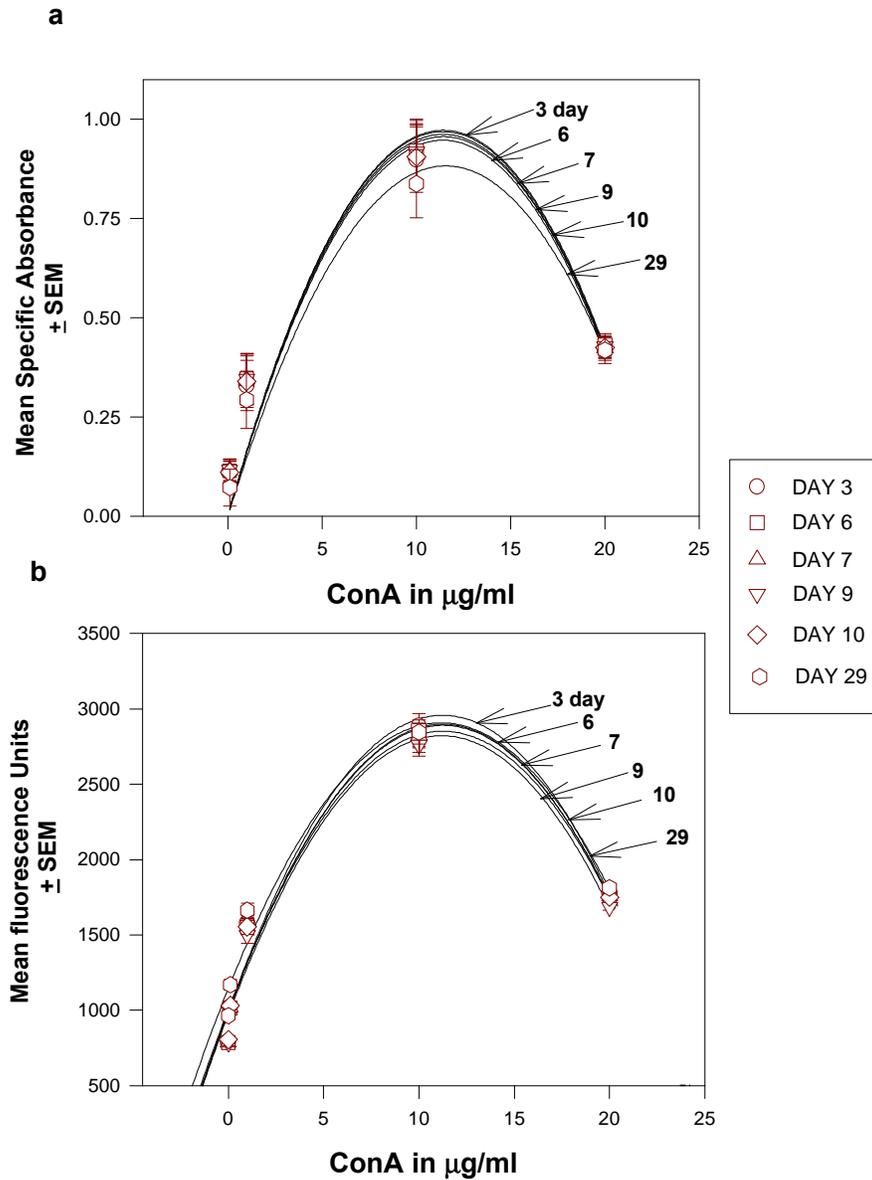
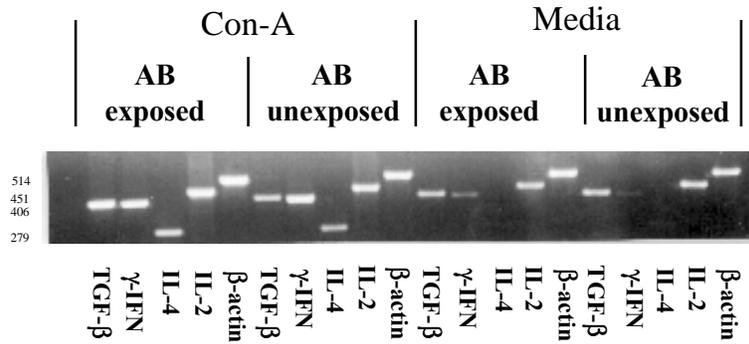


Fig.3.2: Stability of reduced Alamar Blue test. The mitogen-induced proliferation of lymphocytes was assessed by a colorimeter (Fig. 2a) and by a fluorometer (Fig. 2b) after 3 days of culture. The plates were kept in the dark at 4°C and were periodically assessed on the 3, 6, 7, 10, & 29 days after the initiation of culture.

(a)



(b)

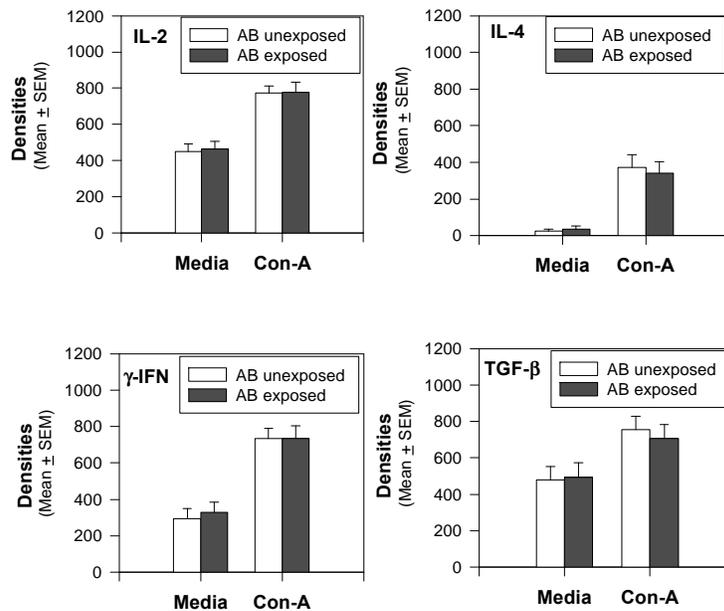
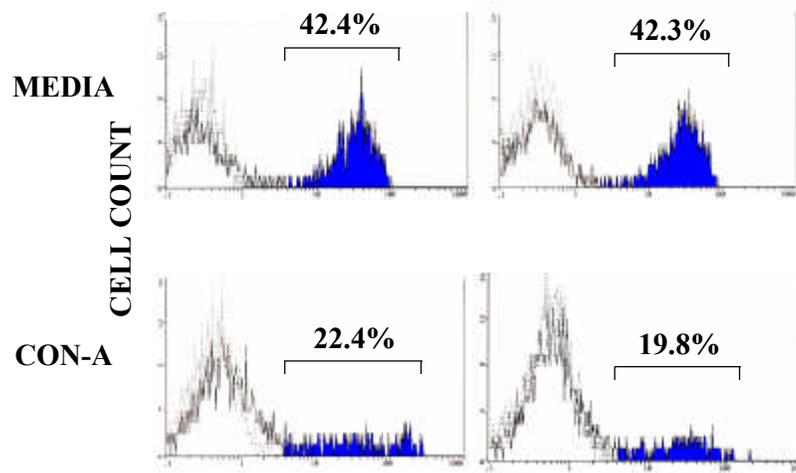
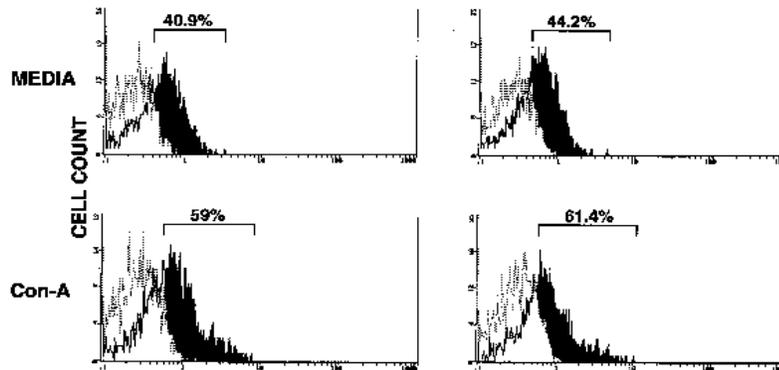


Fig. 3.3: Lymphokine mRNA expression analysis by RT-PCR. RNA was extracted from unstimulated or Con-A-stimulated splenic lymphocytes that were unexposed and exposed to Alamar Blue (AB). Lymphokine mRNA expression was analyzed by RT-PCR using β -actin (internal control), IL-2, IL-4, IFN- γ , and TGF- β primers. The RT-PCR products in the gel were analyzed by scanning densitometer. The top part of the figure shows the photograph of a representative gel. The bottom portion of the figure (histograms) shows mean \pm SEM from five separate experiments. ($P > 0.67$; not significant)

CD90.2



CD2



FLUORESCENT INTENSITY

Fig. 3.4: Analysis of phenotypic characteristics of splenic cells by flow cytometry. Unstimulated (media only) or Con A-stimulated splenic lymphocytes that were unexposed and exposed to Alamar Blue for 24 hours were labeled with FITC anti-CD 90.2 (Thy-1.2) or its isotype FITC-anti-rat IgG_{2a}, κ control antibody; FITC anti-CD2 goat or its isotype FITC-goat anti-rat IgG_{2a}, λ control antibody. Stained cells were subjected to analysis by a Epics-XL flow cytometer.

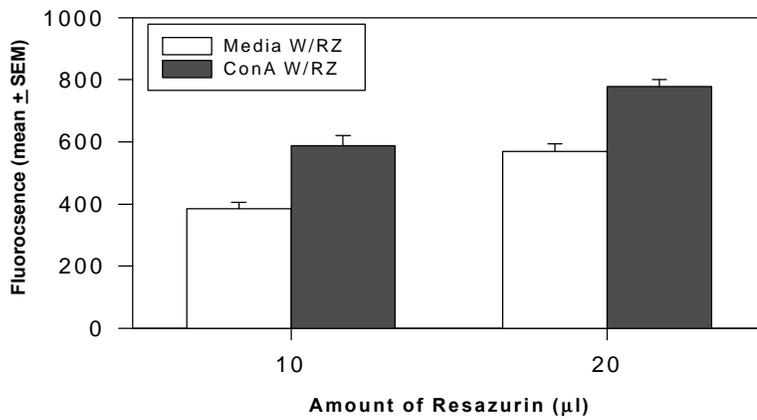
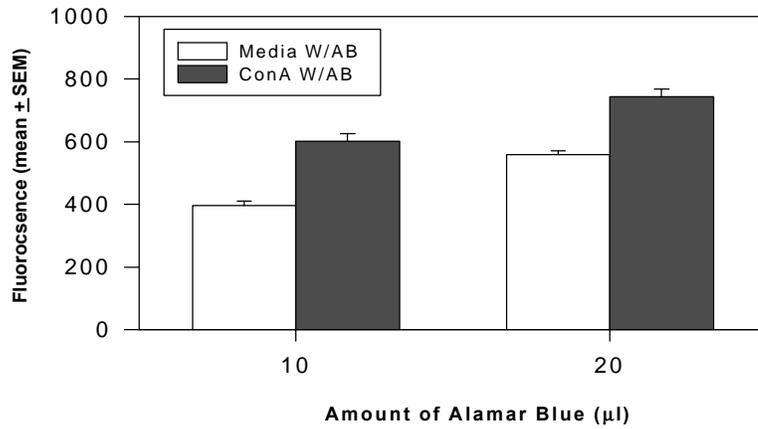


Fig.3.5: Effects of determining lymphocyte proliferation by Alamar Blue and Resazurin assay. Aliquot of splenocytes ($5 \times 10^5/200\mu\text{l}$) were either unstimulated (media only) or stimulated with Con-A and cultured for 72 hours. Ten or 20 μl of Alamar Blue (AB) or Resazurin (RZ) were added during the last 24 hours of culture and the fluorescence units were determined by CytoFluor II ($n = 4$; $p > 0.4$, not significant).

Table I**(a) Cell cycle analysis by propidium iodide assay**

Treatment		Apoptotic (SubG0/G1)		G0/G1		S		G2/M	
	µg/ml	w/o	w/AB	w/o	w/AB	w/o	w/AB	w/o	w/AB
Media		0	0	97.6	94.8	0.1	0.4	1.8	3.7
DEX	0.1	34.4	33.5	44.5	46.6	14.6	12.7	0.8	0.7
	1.0	30.5	30.5	46.8	48.9	15.6	14.1	0.9	0.7

(b) Morphological analysis of apoptosis

Treatment	Apoptotic Index (%)		Necrotic cells (%)		Dead cells (%)	
	w/o AB	w/ AB	w/o AB	w/ AB	w/o AB	w/ AB
Media	40	38	17	20	20	24
DEX (0.1)	54.5	56	27	22	52	45
DEX (1.0)	69	67	22	24	60	65

Table I: Splenic lymphocytes or thymocytes were treated with dexamethasone (DEX: 0.1 or 1.0 µg/ml) for six hours and cells were then exposed (w/) or unexposed (w/o) to Alamar Blue (AB) for an additional 24 hours. Table Ia shows a representative flow cytometric analysis of PI-stained splenic lymphocytes. Table Ib shows thymocytes that were stained with ethidium bromide and acridine orange and the viable, apoptotic and necrotic cells identified under a UV microscope. The above data is a representative of four separate experiments.

Table II

Frequency of chromosomal aberrations in lymphocytes (lymph nodes) of mice exposed to the Alamar Blue dye

Alamar Blue exposure	No. of mice	No. of metaphases	Chromatid Aberrations				Isochromatid Aberrations			Dicentrics	Total no. of aberrations*	No. of polyploids
			Gaps	Breaks	Acentric fragments	Exchanges	Gaps	Breaks	Acentric fragments			
---	4	400	3 (0.75)	7 (1.75)	2 (0.50)	1 (0.25)	4 (1.00)	6 (1.50)	3 (0.75)	4 (1.00)	23 (5.75)	7 (1.75)
+	4	400	2 (0.5)	8 (2.00)	3 (0.75)	1 (0.25)	3 (0.75)	6 (1.50)	5 (1.25)	3 (0.75)	26 (6.50)	6 (1.50)

100 metaphases were analysed for each sample

Values given in parentheses are percentages

* Gaps and polyploids are not included in total number of aberrations

p = not significant

Table II: Lymphocytes from lymph nodes of normal mice were stimulated with PMA plus ionomycin and cultured for 72 hours. Cells were exposed to Alamar Blue during the last 24 hours of culture. Aliquot of cells that were not exposed to the dye served as negative controls. The cells were arrested at the metaphase stage by colchicine. After incubation, the cells were washed twice to remove Alamar Blue. The incubated cells were then subjected to hypotonic treatment and were subsequently fixed using methanol and glacial acetic acid (3:1) fixative. Smears on slides were made and stained with Giemsa and scored for chromosomal aberrations. Chi-Square test was employed to test the validity of the data.

REFERENCES

- Ansar Ahmed, S., Talal, N. and Christadoss, P. (1987) Genetic regulation of testosterone induced immune suppression. *Cell. Immunol.* 104, 91
- Ansar Ahmed, S., Dauphinee, M. J., Montoya, A. I. and Talal, N. (1989) Estrogen induces normal murine CD5-positive B cells to produce autoantibodies. *J. Immunol.* 142, 2647
- Ansar Ahmed, S., Gogal, Jr., R. M. and Walsh, J. E. (1994) A new rapid and non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J. Immunol. methods* 170, 211
- Ansar Ahmed, S., and Sriranganathan, N. (1994) Differential effects of dexamethasone on the thymus and spleen: alterations in programmed cell death, lymphocyte subsets and activation of T cells. *Immunopharmacology* 28, 55
- Ansar Ahmed, S., Gogal Jr, R. M., Larsen C. T. and Pierson, F. W. (1996) A novel non-radioactive diagnostic test for evaluating cell-mediated function in the chicken. *Proceedings XX World's Poultry Congress. Vol. 2*, 533
- Baker, CN., Banerjee, S.N. and Tenover, F.C.(1994) Evaluation of Alamar Colorimetric MIC for antimicrobial susceptibility testing for gram-negative bacteria. *J. Clin.Microbiol.* 32,1261
- Coligan, J.E., Kruisbeek,A.M., Margulies, D.H., Shevach, E.M. and Strober, W. (1994) *Current Protocols in Immunol.* 3.17.1
- Espinel-Ingroff, A., Rodriguez-Tudela, J. L. and Martinez-Suarez, J. V. (1995) Comparison of two alternative microdilution procedures with the national committee for clinical laboratory standards reference macrodilution method M27-P for in vitro testing of fluconazole-resistant and -susceptible isolates of *Candida albicans*. *J. Clin.Microbiol.* 33(12), 3154
- de Fries, Ricarda, and Masato, M. (1995) Quantification of mitogen induced human lymphocyte proliferation: comparison of alamarBlue™ assay to ³H-thymidine incorporation assay. *J. Clin.Lab. Analysis* 9, 89
- Fatima, S. K., Prabhavathi, P. A., Prasad, M. H., Padmavathi, P. and Reddy, P. P.(1995) Frequencies of sister chromatid exchanges in lymphocytes of Portland Cement factory workers. *Bull. Environ. Contam. Toxicol.* 55(5), 704
- Gogal, R. M. Jr., Ansar Ahmed, S. and Larsen, C. T. (1997) Analysis of avian lymphocyte proliferation by a new, simple, non-radioactive assay(Lympho-Pro). *Avian Diseases* (In Press).
- Hujo, H., Tai, L-J., Mukai, T., Hashimoto, Y. (1992) An enzyme immunoassay for cell proliferation using monoclonal antibodies against cell proliferation associated antigen. *J. Pharmacobio-Dyn.* 15: 567
- Huong, P. L.T, Kolk, A. H. J., Eggelte, T. A., Verstinjnen, C. P. H. J., Gilis, H. and Hendriks, J. T. (1991) Measurement of antigen specific lymphocyte proliferation using 5-bromo-deoxyuridine incorporation. *J. Immunol. Methods* 140, 243
- Landegren, U. (1984) Measurement of cell numbers by means of endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cellular surface antigen.*J. Immunol. Methods* 67, 379

Magaud, J. P., Sargent, I. and Mason, D. Y. (1988) Detection of human white cell proliferative responses by immunoenzymatic measurement of bromodeoxyuridine uptake. *J. Immunol. Methods* 106, 95

Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M. and Hungerford, D. A. (1960) Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* 20, 613

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55

Oliveira, S.C., Zhu, Y, and Splitter, G.A. (1994) Recombinant L7/L12 ribosomal protein and g-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4+ T cells. *Immunol.* 83, 1

Page, B., Page, M. and Noel, C. (1993) A new fluorometric assay for cytotoxicity measurements in vitro. *Int. J. Oncol.* 3, 473

Palutke, M., Kukuruga, D. and Tabaczka, P. (1987) A flow cytometric method for measuring lymphocyte proliferation directly from tissue culture plates using Ki-67 and PI. *J. Immunol. Method* 105, 97

Pfaller, M. A., Grant, C., Morthland, V. and Rhine-Chalberg, J. (1994) Comparison evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *Candida albicans*. *J. Clin. Microbiol.* 32(2), 506

Porstmann, T., Ternynck, T. and Avrameas, S. (1985) Quantitation of 5-bromo-2-deoxyuridine incorporation into DNA: an enzyme immunoassay for the assessment lymphoid proliferative response. *J. Immunol. Method* 82, 169

Raza, A., Preisler, H.D., Mayers., G.L, and Bankert, R. (1984) Rapid enumeration of S-phase cells by means of monoclonal antibodies. *New Engl. J. Med.* 310, 991

Robinson, J. P., Darzynkiewicz, Z., Dean, P., Dressler, L., Tanke, H. and Wheelless, L. (1993) *Handbook of Flow Cytometry Methods*. A John Wiley & Sons, INC. Roehm, N.W., G. H. Rodgers, S., Hatfield, M. and Glasebrook, A. L. (1991) An improved colorimetric assay for cell proliferation and viability utilizing tetrazolium salt XTT. *J. Immunol. Methods* 142, 257

Roehm, N.W., Rodgers, G.H., Hatfield, S.M. and Glasebrook, A.L. (1991) An improved colorimetric assay for cell proliferation and viability utilizing tetrazolium salt XTT. *J. Immunol. Methods.* 142, 257

Schmid, I., Uittenbogaart, C. H., Keid B. and Giorgi, J. V. (1994) A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J. Immunol. Methods* 170, 145

Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Current, M.J., Seniff D. and Boyd, M. R. (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* 48, 4827

Squatrito, Robert C., Connor, J. P. and Buller, R. E. (1995) Comparison of a novel redox dye cell growth assay to the ATP bioluminescence assay. *Gynecologic Oncology.* 58, 101

Tiballi, Robert N., He, X., Zarins, L. T., Revankar, S. G. and Kauffman, C. A. (1995a). Use of a colorimetric system for yeast susceptibility testing. *J. Clin. Microbiol.* 33(4), 915

Tiballi, Robert N., Spiegel, J. E., Zarins, L. T. and Kauffman, C. A. (1995b). *Sacchromyces cerevisiae* infections and antifungal susceptibility studies by colorimetric and broth macrodilution methods. *Diagn. Microbiol. Infect. Dis.* 23, 135

Wemme, H., Pfeifer, S., Heck, R. and Muller-Quernheim, J. (1992) Measurement of lymphocyte proliferation: Critical analysis of radioactive and photometric methods. *Immunobiology* 185, 78

Yajko, David M., Madej, J. J., Lancaster, M. V., Sanders, C. A., Cawthon, V. L., Gee, B., Babst, A. and Hadley, W. K. (1995) Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 33(9), 2324

CHAPTER 4

Effect of longterm exposure of normal C57BL/6J inbred male mice to 17 β -estradiol on gene expression in lymphocytes: mRNA analysis of lymphokines and *bcl-2/fas*

ABSTRACT:

It has long been noted that estrogen modulates immune responses. It affects not only antibody-mediated immune responses, but also T cell-mediated responses. In addition, estrogenic hormones are thought to play an important role in pathological autoimmune conditions. In this study, the effects of 17β -estradiol on lymphokine mRNA expression were investigated. Thymocytes, un-separated splenocytes, and splenic T cells were isolated from C57BL/6J male mice, which were orchietomized and treated with 17β -estradiol or placebo for 3-6 months. The isolated lymphocytes were analyzed immediately (*ex-vivo*) as well as upon culture. Cells were cultured in RPMI 1640 complete media alone or in media containing concanavalin A (Con-A 10 μ g/ml) for either 6, 12, and 24 hrs for thymocytes or for 24, 48, and 72 hrs in case of splenocytes. The expression of lymphokine mRNA IL-2, IL-4, IFN- γ , and TGF- β was evaluated by RT-PCR. Using the same method, mRNA expression of proto-onco genes *fas* and *bcl-2* was also investigated. We found that the mRNA expression for lymphokines IL-2 and IFN- γ was significantly increased for Con-A stimulated thymocytes, T-cells and splenocytes ($P < 0.05$) from estrogen-treated mice. TGF- β mRNA seemed to be elevated in both thymocytes and splenic lymphocytes, but the increase was not statistically significant ($P = 0.13-0.91$). Prior estrogen-treatment does not affect IL-4 mRNA in Con-A stimulated thymocytes, un-separated splenocytes, and T-cells compared to placebo treated mice.

17β -estradiol also affects mRNA expression of *fas* and *bcl-2* genes, which regulate apoptosis. Compared to placebo-treated mice, the profile of *fas* for 17β -estradiol treated mice was decreased, while *bcl-2* was increased in thymocytes, T cells and splenocytes. This suggests that 17β -estradiol influences mRNA expression for lymphokine and proto-onco genes. These data may help us in better understanding the mechanisms of estrogen modulation of the normal immune system. The information thus obtained may eventually be useful in understanding female-predominant autoimmune diseases.

Key words: 17β -estradiol (E2), thymocyte, splenocytes, T cells, cytokine, IL-2, IFN- γ , TGF- β , IL-4, Fas, Bcl-2, autoimmune diseases.

INTRODUCTION

Various studies have suggested that sex hormones interact with the immune system contributing either directly or indirectly to the etiology and pathogenesis of various autoimmune diseases. Women are more susceptible to autoimmune diseases. Female-predominant autoimmune diseases include: autoimmune thyroid diseases (Ansar Ahmed et al., 1980; William et al., 1986); systemic lupus erythematosus (Walker et al., 1994), rheumatoid arthritis (Masi et al., 1995), autoimmune diabetes mellitus (Hawkins et al., 1996), idiopathic thrombocytopenic purpura (ITP) (Ahn et al. 1983), Sjogren's syndrome (Apelgren et al., 1996) and multiple sclerosis (Duquette & Girard, 1993). Furthermore, the immune response differs between the sexes. It appears that the female gender group is more susceptible to various autoimmune diseases compared to male. In humans, the susceptibility ratio of female to male in thyroid diseases is 25-50:1; in systemic lupus erythematosus it is 9:1; in rheumatoid arthritis it is 2-4:1; in Sjogren's syndrome it is 9:1; in autoimmune adrenal disease it is 2-3:1; in scleroderma it is 3-4:1; in autoimmune diabetes mellitus it is 5:1 (Ansar Ahmed et al., 1985b, Homo-delarche et al., 1991) and in multiple sclerosis (MS) it is 2-2.5:1 (Duquette et al., 1992). In autoimmune disorder-idiopathic thrombocytopenic purpura (ITP), the clinical diseases is more prevalent in women than in men (Grossman et al., 1984).

Similar sex-related autoimmune diseases were observed in several species of animals. The susceptibility of female rats to experimental autoimmune thyroiditis is higher than in male rats (3:1); and in neonatal thymectomied mice (4:!). Thyroiditis in obese strain of chickens occurs predominantly in female. In systemic lupus erythematosus and rheumatoid arthritis, the disease appears earlier in female than in male mice, including NZB, NZB/NZW F1, NZB/DBA/2 F1, NZB/CBA/N F1, MRL/lpr and MRL/n, and C57BL/6-lpr strains (Ansar Ahmed et al., 1985b). In addition, female NZB/W mice generally die within the first year of life due to glomerulonephritis, while the mortality rate in males at this age is 10% or less (Roubinian et al. 1977; 1978). In immune-mediated polyarthritis, the ratio of females to males is 6:1 in LEW/N rats and 3:1 in LEW/N × F344/N FL rats. Immune mediated hemolytic anemia is higher in female mice than in males. The ratio of autoimmune thrombocytopenia in dog is 2:1 females to males (Ansar Ahmed et al., 1985b).

The precise mechanisms of sex steroid action on cellular immune responses are not well understood. In this study, we focus on how 17 β -estradiol modulates lymphokine mRNA expression to better understand the mechanism of action of this hormone.

Mature T-lymphocytes are divided into two different phenotypic subclasses based on distinctly expressed surface molecules, CD4⁺ or CD8⁺. Both molecules associate with TCR/CD3 complexes. CD4⁺ subclass is restricted by MHC class II and mainly performs "helper" functions so called T-helper cells (T_H). The CD8⁺ subclass is restricted by MHC class I and functions as cytotoxic cells (T_c). T helper cells (T_H) are further divided into T_H1, T_H2 and T_H0 subsets based on their lymphokine profile (Mosmann, 1996). T_H1 cells produce IL-2, IFN- γ and lymphotoxin (LT). These lymphokines are not detectable in production of T_H2 subset. T_H2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. T_H0 cells produce lymphokines produced by T_H1 and T_H2. All subset are capable of secreting TGF- β (Mosmann et al., 1989, Gilmore et al., 1997). The T_H1 subset functions predominantly in inflammatory and cell-mediated immune responses. The T_H2 subset helps B cells to produce immunoglobulin in humoral immune responses, and also promotes anti-inflammatory function (Gilmore et al., 1997).

During T cell proliferation, the initial signal which triggers the T cell activation is that TCR/CD3 complex on the T cell surface, which binds with foreign antigen associated with self MHC. The second signal--costimulatory signal initiated by binding of CD28 on the T cell to B7 molecules on the APC is also needed. As the binding signal transfers through the TCR/CD3 complex, early membrane and cytoplasmic events occur. These include: (1) tyrosine phosphorylation of membrane and cytoplasmic proteins; (2) plasma membrane inositol phospholipid hydrolysis; (3) increases in cytoplasmic calcium concentrations, and (4) an increase in protein kinase C activity (Ullrich et al., 1990; Abbas et al., 1994). These changes lead to transcriptional activation and expression of genes, such as cellular proto-onco gene/transcription factor genes, lymphokine, and lymphokine receptor genes, followed by translation.

Another mechanism of regulating immune responses is to control lymphocyte cell death by apoptosis or programmed cell death (PCD). It is an active form of cell death which meets the requirement for proper development or function of an organ and system. PCD can be distinguished from necrosis based on morphology and biochemical features. The cell undergoing PCD shows shrinkage, chromatin condensation and formation of apoptotic bodies. DNA is degraded into multiple fragment which reveal a "ladder" pattern on agarose electrophoresis (Cohen et al., 1992, Wyllie 1993). PCD involves in maturation of cells in the immune system and mediates many important functions not only in humoral immune responses but also in cell-mediated immune responses. During T-cell maturation, the positive and negative selections are achieved by PCD, which eliminate 97% of nonfunctional T-cells including autoreactive T-cells. In immune responses, PCD plays an important role in eliminating active T-cells and terminating the immune response following successful clearance of antigen (Duke et al., 1986).

Two critical molecules involved in apoptosis are Fas and Bcl-2. Fas antigen induces cell death through PCD. One of the important roles of Fas-mediated apoptosis in the immune system is the elimination of autoreactive T-cells. Bcl-2 plays an opposite role. It inhibits apoptosis and provides a signal for maintaining cell survival (Korsmeyer et al., 1992).

In this study, thymocytes, un-separated splenocytes, and enriched T-cells were isolated from orchietomized C57BL/6J male mice which had been implanted with 17 β -estradiol or placebo implants (PO). The expression of lymphokine mRNA's IL-2, IL-4, IFN- γ , TGF- β , and proto-onco genes *fas*, and *bcl-2* mRNA's was investigated in order to explore the effects of 17 β -estradiol on the cell-mediated immune response.

Materials and methods:

Animals: Three-week-old, thirty-two C57BL/6J inbred male mice were purchased from Charles River Laboratory. These mice were fed a commercially available pelleted diet and were maintained in our Center for Molecular Medicine & Infectious Diseases (CMMID) laboratory animal facility under 12 hours light and 12 hours dark condition.

Silastic implants: An eight-mm length of silastic medical grade tubing (internal diameter 0.062" \times 0.125" outer diameter) was prepared. Six to eight mg of 17 β -estradiol (Sigma Chemical Co., MO) powder was placed inside the silastic tubing. The tubing was then sealed at both ends using silastic medical adhesive (silicone type A, Dow Corning Corp., Midland, MI). Empty tubing was prepared similarly and was used for the placebo control group (Ansar Ahmed et al., 1986, 1989). All procedures were performed under sterile conditions.

Orchiectomy and sex hormone treatment: Four to six-week old mice weighing 14.0-15.0g were orchietomized under sodium pentobarbital (embutal) anesthesia (0.13-0.14 μ g/g body weight). At the same time, one sterile 17 β -estradiol implant or empty (placebo) implant was surgically placed subcutaneously on the lumbar dorsum of each mouse. Silastic 17 β -estradiol implants release hormone slowly for several months to maintain serum concentrations of 425.3 \pm 29.1pg/ml (Roubinian et al., 1978, Ansar Ahmed et al., 1986). Serum concentrations of 17 β -estradiol in orchietomized mice with placebo's implant was 66.8pg/ml \pm 5.2pg/ml (for detail see table 4.1) (Ansar Ahmed et al., 1989). The surgical procedure was performed under aseptic conditions.

Method of sacrifice: 17 β -estradiol treated and placebo treated mice were sacrificed by cervical dislocation after 3-6 months of the above treatment, according to procedures approved by our institutional animal welfare guidelines.

Isolation of Lymphocytes. Spleen and thymus were removed from mice under sterile conditions and tissue were gently teased on a metallic sieve screen (Sigma, St. Louis, MO) as described in earlier studies (Ansar Ahmed et al., 1987, Ansar Ahmed et al., 1989) to obtain single cell suspension in RPMI 1640 media (Mediatech., Cellgro, Herndon, VA). Lymphocytes were isolated by either ACK-lysis or by Lympholyte $\text{\textcircled{R}}$ -M methods. Lymphocytes were washed twice in RPMI 1640 media (centrifugation 250 \times g, 4 $^{\circ}$ C, 6 minutes). Erythrocytes in the cell pellet were eliminated by incubating with 1ml of ACK-lysis buffer (pH 7.2) at room temperature for 3 minutes, followed by washing at least twice with fresh RPMI media immediately as described (Ansar Ahmed et al, 1989, 1994). The cell-suspensions were adjusted to 5 \times 10⁶ cells /ml in complete RPMI media supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, GA), 2mM of L-glutamine, 1% non-essential amino acids, 50 IU/ ml of penicillin and 50 μ g/ ml of streptomycin (INC pharmaceuticals. Inc. CA).

Alternatively, unseparated splenic cells were incubated at 37 $^{\circ}$ C for 10-15 minutes. They were then carefully layered (no more than 5 \times 10⁸ cells) onto 5 ml of Lympholyte $\text{\textcircled{R}}$ -M (Accurate Chemical & Scientific Corp. NY) at room temperature. The samples were centrifuged at 1,500 \times g at room temperature for 25 minutes. Lymphocytes remained at interface between the top pink layer and the lower clear layer (Fig. 4.1) after centrifugation. Lymphocytes were collected and washed three times with 5 ml of RPMI media and were counted using a hemocytometer to determine concentration. This method of purification yielded a cleaner preparation of lymphocytes compared to ACK-lysis by far.

Enrichment of T cells:

Splenic T cells were enriched initially by negative selection using complement and subsequently by positive selection using a magnetized columns. (1). Un-separated splenic cells from the ACK-lysis method were resuspended in cold RPMI media (10⁷ cells/ml). B cells and residual RBCs were targeted by anti-heat-stable antigen monoclonal antibody, while macrophages and B cells were targeted by anti-heat-class II antibody. One ml of straight supernatant of anti-stable antigen monoclonal antibody (from cell line J11d.2) and anti-class II (I-A^{b,d}) antibodies (anti-I-A^b antibody from cell line 25-9-17SII, anti-I-A^d antibody from cell line MK-D6) (Ansar Ahmed's Lab) was added to 10⁷ cells and incubated with gentle shaking at 4 $^{\circ}$ C for 1 hour. The cells were washed twice with cold RPMI media at 4 $^{\circ}$ C, 250 \times g, for 6 minutes. One ml of 1:20 diluted

complement (Pel-Freez® Biologicals, AK) (dilution performed in serum-free room temperature RPMI media) was added to 10^7 cells. The cells in the complement solution were incubated at room temperature with gentle rocking for 15 minutes, followed by washing twice immediately with cold RPMI media. These purified T cells were re-suspended in complete RPMI media at 5×10^6 cells/ml.

(2). Isolated un-separated splenic cells (10^7) from the Lympholyte®-M method were resuspended in 90 μ l of MACS buffer (PBS with 0.5 mM EDTA, AND 0.5% BSA). The target population of cells was determined approximately by multiplying total number of lymphocytes by 0.35 (approximate % of T cells in the spleen). Ten microliters (μ l) of MACS anti-CD90 (Thy 1.2) (Miltenyi; Biotec, Germany) antibodies was added to 10^7 target cells and mixed. The samples were incubated at 6-12°C for 15 minutes. MACS separation columns (Fig. 4.2) were washed with 5 ml of MACS buffer, then cells labeled with MACS anti-CD90 were added to the column reservoir. Effluent was collected as the negative fraction. MACS columns were then washed three times with 3 ml of MACS buffer slowly. Five ml of Macs buffer were then added to MACS column and T cells were carefully flushed from the column using the column plunger.

Incubation of cells: One hundred microliters of un-separated splenocyte, enriched T cell and thymocyte suspension (5×10^6 cells/ml) were placed in 96-well sterile culture plates (Corning, NY) containing 100 μ l of complete RPMI media or 100 μ l concanavalin A (Con-A 10 μ g /ml). The plates were incubated at 37°C (5% CO₂) under humidified conditions for various intervals (6, 12, and 24 hours for thymocytes; 24, 48, and 72 hours for un-separated splenocytes and enriched T cells).

Cell viability assay. Cultured un-separated splenocytes, thymocytes and T cells were examined for the cell viability using trypan blue exclusion-method (Ansar Ahmed et al., 1987) at different incubation time points.

Total RNA extraction. In order to avoid contamination with ribonuclease during extraction of RNA, all glassware was baked at 180°C for 8 hours, aerosol resistant RNase-free filter tips were employed and all solutions were treated with 0.01% DEPC (diethyl pyrocarbonate). Latex gloves were worn during the entire procedure.

Un-separated splenocytes, enriched T cells, and thymocytes were transferred into autoclaved microcentrifuge tubes (cells in each well/tube). Five hundred microliters of TRIzol™ reagent (Life Technologies, Inc. MD) was added to each tube. The cells were then homogenized and incubated for 10 minutes at room temperature. One hundred microliters of chloroform was added to deproteinize the sample. The samples were gently mixed and incubated at room temperature for 15 minutes followed by centrifugation at $12,000 \times g$, 4 °C for 15 minutes (MTX-150, Peninsula Laboratories, Inc. CA). The upper aqueous phase containing the RNA was transferred to a fresh microcentrifuge tube (excluding the interface), and 250 μ l of isopropyl alcohol was added to precipitate total RNA, the mixture was incubated at room temperature for 10 minutes, followed by centrifugation at $12,000 \times g$ at 4 °C for 15 minutes. The total RNA pellets were washed with 0.5 ml of 75% cold ethanol and were pelleted again by centrifugation at $12,000 \times g$, 4 °C for 15 minutes. The RNA samples were stored at -70 °C until use.

Cytokine mRNA analysis. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect β -actin, IL-2, IL-4, IFN- γ , TGF- β_1 , *fas*, and *bcl-2* mRNA expression. For the synthesis

of cDNA, total RNA from 5×10^5 cells was suspended in a 40 μ l reaction solution containing 2.0 μ g of oligo(dT)₁₂₋₁₈ (GIBCO BRL, Gaithersburg, MD) 1.25 mM of dNTP, 20.0 units of RNasin (Promega Corporation Madison, WI), 1 \times RT buffer, 4.0 μ l of 0.1M DTT and 1.5 μ l (300 units) of moloney murine leukaemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The reaction mixture was incubated at 37°C for 1 hour. The PCR reactions were performed in 25 μ l of volume, containing 5 μ l of the cDNA samples from the previous step. The reaction mixture included: 2.5 mM MgCl₂, 0.25 mM of dNTP, 1 \times PCR buffer, 1 μ M each specific primer, and 0.2 μ l (1.0 units) of Taq DNA polymerase (GIBCO, BRL, Gaithersburg, MD). The PCR reactions were performed using a Omnigene TR3 CM110 thermocycler (Hybaid Limited, TW) under the following conditions: 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and amplification at 72°C for 1.5 minutes. A final extension was done for 10 minutes at 72°C. Twelve microliters of PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide (0.45 μ g/ml) at 75 volts. Electrophoresis was stopped when the dye front reached two-thirds the distance from the origin to the end of the gel. For analysis of cytokine mRNA expression, photographs were taken and the RT-PCR products were evaluated using scanning densitometry (Molecular Dynamics, CA).

Oligonucleotides used for PCR: Specific primers of β -actin, IL-2, IL-4, IFN- γ , and TGF- β for mouse were purchased from the Stratagene Company, CA. Primers for *fas* and *bcl-2* were designed using DNA-star software:

(1) Fas :

(a) Sense primer

5'-AGGCCGCCCCGCTGTTTTCCC-3'

(b) Antisense primer

5'-CCATCATGGGTGGCAGGC-3'

(2) Bcl-2:

(a) Sense primer

5'-CCTGCAGCTTCTTTTCGGGG-3'

(b) Antisense primer

5'-GCATATTTGTTTGGGGCAGG-3'

Statistical analysis. The student' *t*-test were employed to assess the significance of the densitometric data relative to cytokine mRNA and proto-onco gene mRNA expression. The data are presented as mean \pm SEM. $P < 0.05$ was considered significant.

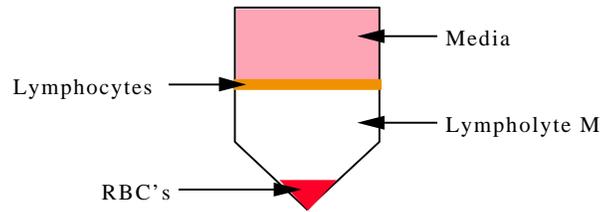


Figure 4.1. Lymphocytes were isolated using Lympholyte[®]-M. Unseparated splenocytes (less than 5×10^8 cells) were layered onto 5 ml of Lympholyte-M at room temperature, followed by centrifuge. Lymphocytes migrated as depicted in Fig.4.1. Media remained at top, lymphocytes resided below media and above the Lympholyte-M solution. Erythrocytes were pelleted at bottom.

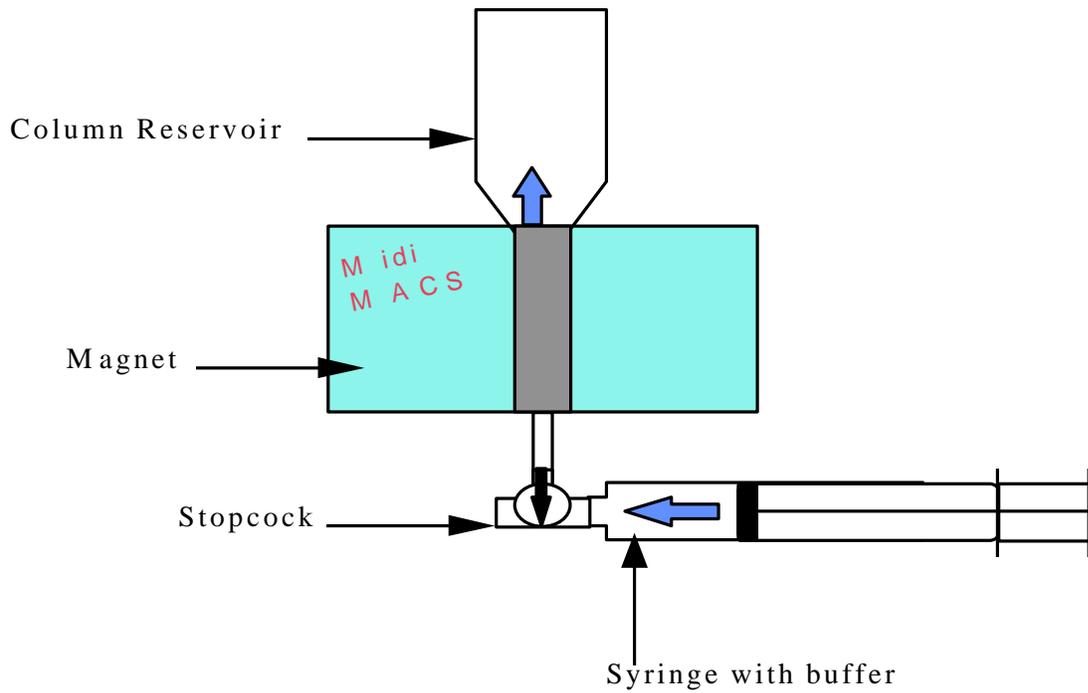


Figure 4. 2. Schematic of MACS LS+ separation column system. Unseparated splenocytes were incubated with MACS anti-CD90 and were added to the column reservoir. The labeled T lymphocytes were held in the column by the MACS magnet during washing. T cells were then eluted from the column by washing with MACS buffer and were flushed out using the column plunger.

RESULTS

Effects of 17 β -estradiol on cell viability

Thymocytes, enriched T-cells, and un-separated splenocytes isolated from placebo (PO) mice and 17 β -estradiol (E2) treated mice were cultured in media alone (unstimulated cells) or in mitogen, Con-A (10 μ g/ml). The same number of cells (5×10^5 /well) were used in each of the experiments to start with and were incubated at 37°C, 5% CO₂ under humidified condition for various periods. The cell viability was examined using the trypan blue exclusion assay before extraction of RNA.

Thymocytes: Thymocyte viability (expressed as a percent of control cells) was examined following 0, 6, 12, and 24 hours of cultivation. The viability of cells from E2-treated mice was decreased at all time points compared to control group (PO) cells. It was decreased by 22%, 44%, and 53% in media alone; and decreased by 41%, 41%, and 60% in Con-A at 6, 12, and 24 hours, respectively (figure 4.3 A) (n = 6).

Splenocytes: Un-separated splenocyte viability was examined at 0, 24, 48, and 72 hours cultivation. The viability of cells from E2-treated mice was decreased at all culture time points in comparison with mice of control group (PO). It was decreased by 16%, 18%, and 21% in media; and by 39%, 41%, and 31% in Con-A at 24, 48, and 72 hours, respectively (figure 4.3C) (n = 6).

Enriched splenic T-cells: Similarly enriched T cell viability was examined at 0, 24, 48, and 72 hours cultivation. In controls, numbers of viable T cells increased by 24 hrs followed by a gradual decrease. However, the viability of cells from E2-treated mice was decreased at all culture time points in comparison with control group (PO). It was decreased by 52%, 59%, and 55% in media; and by 57%, 48%, and 60% in Con-A at 24, 48, and 72 hours, respectively (figure 4.3 B) (n = 6). By comparing thymocyte, splenocyte and T cell, T cell and thymocyte were more sensitive to E2 than splenocytes. That is the decrease in viability was more profound for T cells and thymocytes than for splenocytes.

Effects of 17 β -estradiol on lymphokine mRNA expression

RNA was extracted from thymocytes, splenocytes, and T cells in both control (PO) and E2-treated mice. RT-PCR was employed for the evaluation of mRNA expression. In order to study the effects of E2 in possibly altering T_H1 and T_H2 balance, mRNA of the following lymphokines IL-2, IFN- γ , TGF- β , and IL-4 were examined. β -actin was used as internal cell control.

Thymocytes: Lymphokine mRNA expression was examined at 0, 6, 12, and 24 hours of culture. Compared to the control group (PO), E2 enhanced IL-2 mRNA expression in media alone at all time points in this study (P = 0.22-0.43), and in response to Con-A stimulation at 12 and 24 hours (significant P = 0.003-0.006, Fig. 4.4]. E2 increased IFN- γ mRNA expression in media culture condition at all time points, but not statistically significant (P = 0.12-0.44). However, upon Con-A stimulation there was a significant increase in IFN γ mRNA expression at 12, and 24 hours (P = 0.013, 0.010) respectively (Fig. 4.5). TGF- β was not affected in media alone at any time points (P = 0.56-0.83), but appeared to increase in response to Con-A stimulation at 6, 12 and 24 hours (not statistically significant, P = 0.91, 0.19, and 0.13 respectively). As cultivation time increased, effects tended to be significant (Fig. 4.6). IL-4 mRNA expression was not affected by E2 treatment either in media alone (P = 0.57-0.93) or in response to Con-A stimulation (P = 0.42-

0.98) (Fig.4.7). Overall lymphokine mRNA expression (IL-2, IFN- γ , TGF- β and IL-4) of thymocytes by treatment of E2 was shown in figure 4.22.

Splenocytes: Lymphokine mRNA expression was examined at 0, 24, 48, and 72 hours. E2-treated cells were compared with control group (PO) cells, E2 did not affect IL-2 mRNA expression in media alone at any time points, but positively affected the IL-2 expression in response to Con-A stimulation at 48 hours (P = 0.03) (Fig. 4.12). IFN- γ mRNA expression was not affected by E2 treatment in un-stimulated cells at any time points, but was significantly increased in response to Con-A stimulation at 24 hours (P = 0.03). At 48 and 72 hours, IFN- γ mRNA expression was not significantly increased in stimulated cells compared to control group (PO) cells (P = 0.08 and 0.68) (Fig. 4.13). TGF- β was enhanced both in media alone (P = 0.49, 0.36, 0.31, and 0.09 at 0, 24, 48, and 72 hours respectively) and in response to Con-A stimulation at all time points (P = 0.25, 0.18, and 0.24, at 24, 48, and 72 hours respectively) (Fig. 4.14). IL-4 mRNA expression was not affected by E2 either in media alone or in response to Con-A stimulation (P = 0.50-0.99) (Fig.4.15). Overall expression of lymphokines (IL-2, IFN- γ , TGF- β and IL-4) of splenic lymphocytes by treatment of E2 was shown in figure 4.24.

Enriched splenic T cells: Lymphokine mRNA expression was examined at 0, 24, 48, and 72 hours. Compared to control group (PO), E2-treated mice appear to have no effect on IL-2 mRNA expression in media alone at all time points, but positively affected the IL-2 mRNA expression in Con-A-stimulated cultures at 48 hours (P = 0.04) (Fig. 4.8). Although IFN- γ mRNA appeared to be increased for enriched T cells, results were not statistically significant (P = 0.20-0.91) in media alone. IFN- γ mRNA was significantly increased in response to Con-A stimulation at 48, and 72 hours (P = 0.04, and 0.048) (Fig. 4.9). TGF- β was increased in both in media alone (P = 0.23-0.86) and in response to Con-A stimulation (P = 0.28-0.58) at all time points, but did not reach the level of significance (Fig. 4.10). IL-4 mRNA expression was not affected by E2 treatment either in media alone (P = 0.34-0.78) or in response to Con-A stimulation (P = 0.60-0.77) (Fig.4.11). Overall expression of lymphokines (IL-2, IFN- γ , TGF- β and IL-4) of enriched splenic T cells by treatment of E2 was shown in figure 4.23.

Effects of 17 β -estradiol on apoptotic gene *fas* & *bcl-2* mRNA expression

In order to study the influence of E2 on apoptosis, the expression of proto-onco gene *fas* and *bcl-2* mRNA were measured in thymocytes, un-separated splenocytes, and enriched T cells. E2 treatment changed the profiles of *bcl-2* and *fas*, but these changes were not statistical significant.

Thymocytes: *bcl-2* mRNA expression was increased at all time point in media alone and under Con-A stimulation. The increase in expression became more profound as culture time was prolonged. The increase ranged from 17-151% in media alone, and from 3-100% in response to Con-A stimulation (Fig. 4.16). On the other hand, *fas* mRNA was decreased in E2-treated thymocytes both in media alone and under the influence of Con-A at all time points. The range of these changes was 4-19% in media alone, and 9.3-37% under Con-A stimulation (Fig. 4.17).

Splenocytes: *bcl-2* mRNA expression was apparently increased in media at the most of culture time points, increased in response to Con-A at all culture time points. The increase ranged from 9.2 to 25.2% (Fig. 4.20). *fas* mRNA was decreased early in the cultivation period, but was

increased at later intervals in media alone. *fas* mRNA was decreased at 24 hours, but was not affected at 48 and 72 hours in response to Con-A stimulation (Fig. 4.21).

Enriched splenic T cells: *bcl-2* mRNA expression was not affected in T cells cultivated in media alone, but was higher for E2-treated T cells in response to Con-A at all time points. Although the range of increase was 51-74%, this did not represent a level of significance (Fig. 4.18). *fas* message was slightly decreased by E2 treatment at some culture times in media alone. It was decreased at all time points in culture with Con-A stimulation, the range of decrease by E2 treatment was 11.2-31.8% (Fig. 4.19).

DISCUSSION

In this series of studies, we first investigated the effect of E2 on lymphocyte viability at different culture periods. The data clearly demonstrate that lymphocytes are sensitive to the influence of 17 β -estradiol, especially thymocytes and T cells. Survival of thymocytes and splenic T cells from E2-treated mice was obviously reduced (22-53%, 52-59% in the media alone, 41--60%, 48-60% with Con-A stimulation compared to that from PO mice at 24 and 72 hours respectively). The viability of splenocytes from E2 treated mice was reduced by 16-21% in media alone, and 31-41% in Con-A within 72 hours.

Next we investigated the effects of E2 on lymphokine mRNA (IL-2, IFN- γ , TGF- β 1, and IL-4), and proto-onco gene *fas* & *bcl-2* mRNA. We found that E2 mediated various lymphokine mRNA expression in thymic and splenic lymphocytes. It also modulated proto-onco gene mRNA expression. Our data showed that E2 increases the mRNA expression of T_H1 subset (IL-2, IFN- γ , and TGF- β 1), but does not affect T_H2 subset (IL-4) of the T_H population. E2 also appear to modulate genes that regulate apoptosis. There was an apparent increase in *bcl-2* mRNA and a decrease in *fas* message in thymocyte, splenic T cell and splenocytes of E2-treated mice.

Among the lymphokines, IL-2 is considered the major T cell growth factor produced by the T_H1 subset of T_H cells. It is the only known T cell factor that moves the T cell from early G1 phase into the S phase of the cell cycle (Hassuneh, 1996). Following T cell activation, the cell secretes its own growth-promoting lymphokine IL-2 and also expresses the cell surface receptor for this lymphokine. As the result, IL-2 performs autocrine function on some T cells, and paracrine function on adjacent cells in order to stimulate cell proliferation. IL-4 and IL-5 are considered the primary growth factors for B cells. In addition to stimulating B cell growth, they also stimulate B cell immunoglobulin production and functions of T cells.

In our experimental system, one paradoxical finding resulted. Even though IL-2 mRNA was increased in remaining lymphocytes from E2-treated mice, the viability of these IL-2 producing cells was lower than placebo-treated cells. As we mentioned above, IL-2 induces T cell mitotic division. (To understand this phenomenon, based on the lymphokine mRNA levels that was investigated in this study.) We considered that there could be two outcomes for IL-2 production, either a correlated or an un-correlated to mRNA product. Therefore, four possibilities exist to explain this contradictory phenomenon: (1) E2 impacts enzymes or interferes with steps at the level of translation. Even though mRNA synthesis is higher for cells from E2-treated mice, IL-2 protein synthesis may be blocked or disrupted. (2) IL-2 receptors on the T cell surface are conformationally changed or mutated by long-term exposure to E2. It had been

documented that E2 treatment increases the amount of IL-2 in cell culture supernatants (Magnusson, 1991, Viselli et al., 1995). To test this hypothesis, examination of the IL-2 protein levels in supernatant and detection of IL-2 receptors on T cell surface need to be performed simultaneously. (3) It is also possible that IL-2 binds with soluble receptors or receptors in cytoplasm instead of the receptors on cell surface. IL-2 protein might not exert normal functions for this reason, because of inhibition of the activity of IL-2 protein. Even though the mechanism of E2-mediated lymphocyte proliferation is not fully understood, reports indicate that estrogen can interfere with the pathway controlling cell division and with synthesis of structures of the cytoskeleton (Wheeler et al., 1986; Tucker et al., 1986; Epe et al., 1989). The fourth reason we assume that 17 β -estradiol binding to its own receptors in lymphocytes, stroma cells or connective tissues directly or indirectly influences cell metabolism, which impact early membrane/intracellular signal events in lymphocyte activation, such as decreasing phosphorylation and changing cytoplasmic calcium concentration that skews the cell development leading to decrease of lymphocyte viability.

Comparing the viability of splenocytes with thymocytes and splenic T cells, the influence of E2 in splenocyte viability is less profound than in thymocytes and splenic T cells. The reason may be that E2 produces relatively hyperactive effect on B cells. It has been clearly demonstrated by others that mice treated with E2 have high levels of antibodies and autoantibodies; the activity of B cell was relatively increased, while total lymphocytes were decreased; and B cell cycle was increased (data were not shown).

It has been previously reported that estrogen promotes B cell functions. Women have higher plasma IgG and IgM levels than men (Olsen et al., 1996). Females mount a greater immunoglobulin response to immunization (Ansar Ahmed et al., 1990). On the other hand, administration of estrogen and estrogen analogues to normal or autoimmune mice induce autoantibody production, including anti-dsDNA, anti-cardiolipin (CL), anti-phosphatidylinositol (PI), anti-phosphatidylserine (PS), anti-phosphatidylcholine (PC), and anti-phosphatidylethanolamine (PE) (Roubinian et al., 1978, Carlsten et al., 1992, Ansar Ahmed et al., 1993a, 1993b, Verthelyi et al, 1994,). In a few reports, it has been demonstrated that estrogen also modulates cell-mediated immune responses by influencing lymphokine profiles. In multiple sclerosis patients, 17 β -estradiol increased the production of IFN- γ and IL-10. TNF- $\alpha\beta$ production showed biphasic response to E2. TNF- $\alpha\beta$ was increased at the low dose of E2, and decreased at high dose. In the same experimental system, E2 had no effect on the secretion of IL-4 and TGF- β_1 from T cell clones (Gilmore et al., 1997). Fox et al. (1991) isolated un-separated spleen cells from CD-1 mice, followed by treatment of the cells with 17 β -estradiol and incubation with Con-A. They found that the IFN- γ was produced at sustained high levels. Short-term exposure to estradiol also increased IFN- γ mRNA expression in Con-A-treated murine spleen cells. IL-2 and IFN- γ lymphokine production by Con-A stimulated un-separated spleen cells was increased in castrated normal C57BL/6J male mice compared to intact male mice (Viselli et al., 1995). Overall these results suggest that estrogen enhance IFN- γ and IL2, while male hormone have opposite effect.

In our study, we found that mRNA expression of IL-2 and IFN- γ was significantly increased, and that TGF- β_1 was increased but not significantly, while IL-4 mRNA was not

affected in response to Con-A stimulation in thymocytes, splenic T cells, and splenocytes from orchietomized C57BL/6J mice, following long-term exposure to 17 β -estradiol. The data for IL-2, IFN- γ and IL-4 from our results agree with that of other researchers. The difference of TGF- β_1 profiles may be because of different experimental system, different animal genetic background, different levels of expression, cell specificity or duration of E2 exposure period .

T cells play a major role in immune responses. Under normal conditions, T_H2 type response predominates (Abbas et al., 1994). In autoimmune diseases, cytokine profiles are complex. It has been shown that an unbalanced or skewed expression of T_H1 and T_H2 lymphokine has been associated with autoimmune diseases. T_H1 cells are thought to be involved in the induction of autoimmune diseases. In SLE (Huang et al., 1988), PSS (Kahaleh et al., 1989), and MS (Adachi et al., 1989), serum IL-2 levels were abnormally high (as determined by means of bioassays or commercial enzyme-linked immunoassays). IFN- γ is also capable of facilitating induction or aggravating manifestations of autoimmune diseases such as EAE (Mustafa et al., 1991), autoimmune arthritis (Billiau, 1996), and autoimmune insulinitis (Sarvetnick et al., 1990). IFN- γ and IL-2 mRNA expression are significantly increased before the onset of autoimmune lesions observed in Sjogren's syndrome of MRL/lpr mice, a condition that resembles human SS and SLE (Hayashi et al., 1996). Shirai et al. (1995) observed the IL-4 and IFN- γ profiles with same model compared to that MRL \pm/\pm and BALB/c mice by ELISpot assay. They found that the ratio of IFN- γ : IL-4 was increased, but it was decreased in normal animals in the spleen, mesenteric lymph nodes, bone marrow and peritoneal cavity cells. IL-1, IL-5, IL-6, IFN- γ , TNF- α , TNF- β , and TGF- β mRNA expression were increased in spleen, lymph nodes, peripheral blood or inflamed tissues (Mori, et al., 1994). Experimental allergic encephalitis is associated with a predominant T_H1 pattern of lymphokine expression (Chen et al., 1994, Racket et al., 1994, Kuchroo et al., 1995). CD4⁺ T cell clones isolated from lymphocytic infiltrates of Hashimoto's thyroiditis and Grave's disease exhibit a clear-cut T_H1 phenotype (De et al., 1993). In the other studies of insulin dependent diabetes mellitus (IDDM), the high level of IL-10 contributed to the β -cell destruction (Wogesen, et al., 1994, Anderson et al., 1993). Cytokine expression in synovial fluids of RA human, or animal model appears that monocytes produce IL-1, IL-6, IL-8, IL-10 and TNF α (Ivashkiv, 1996). IL-2 products are also detected in the synovial fluids of RA patients (Lemm and Warnatz, 1986). IL4 protein levels in synovial fluids are very low or undetectable using an ELISA (Miossec et al., 1990). Some arthritis associated with caprine arthritis-encephalitis virus (CAEV), appears to involve a T_H2 type of response (Cheevers et al., 1994).

The increase in IL-2, IFN- γ , and TGF- β_1 by long-term E2 treatment correlated with changes that occur with some autoimmune diseases. This suggests that 17 β -estradiol mediates a selective lymphokine response of the T_H1 phenotype, this establishing a relationship between autoimmune diseases and E2 exposure. E2 increases T_H1 cell type activity, which may contribute to systemic and organ-specific lesions in autoimmune disorders.

Apoptosis is responsible for thymic negative selection, which causes a massive loss of thymocytes and the establishment of immunologic tolerance. It is important for normal immune system development and for appropriate immune responses. Fas and Bcl-2 are the products

involved in apoptosis, and these molecules directly target cell's death. Fas, a membrane-associated polypeptide, transduces a death signal to the cell by binding to the ligand FasL. This binding in turn blocks cell growth and induces apoptosis of various human T and B cell lines *in vitro* (Trauth et al., 1989) and *in vivo* (Ogasawara et al., 1993). Bcl-2 is associated with the mitochondrial membrane. It enhances lymphoid cell survival by interfering with apoptosis rather than promoting cell proliferation (Vaux et al., 1988). In both normal and neoplastic tissues under experimental conditions, expression and overexpression of the *bcl-2* gene product appears to protect cells from death by preventing or delaying apoptosis.

Recent studies reported that Fas and Bcl-2 are involved in autoimmune diseases. The MRL lpr/lpr mouse strain is a model of human SLE and SS autoimmune diseases. The characteristic of lpr (lymphoproliferation) mouse is a defect in the cell-surface receptor, Fas, that mediates apoptosis. The defect is characterized by lymphadenopathy, autoimmune syndrome and production of multiple circulating autoantibodies. Several published papers indicate that a defect in negative selection of self-reactive thymic T lymphocytes, results in the release of excessive number of self-reactive T cells into the peripheral circulation. This prevents the self-tolerance mechanism (Ogawa et al., 1995, Rose et al., 1994, Drappa et al., 1993). Patients that suffer from Hashimoto's thyroiditis (HT) exhibit abundant *fas* mRNA expression in HT glands (Giordano et al., 1997) which accelerates apoptotic cell death to clinical hypothyroidism. In human SLE cases, isolated T and B cells show significantly higher *fas* expression compared to normal controls (Mysler et al., 1994). In addition, SLE lymphocytes demonstrate accelerated apoptosis *in vitro*. This phenomenon is also observed in Sjogren's syndrome (SS) patients (Emlen et al., 1994).

Bcl-2 is also an important molecule involved in apoptosis, immune regulation and autoimmune diseases. Graninger (1993) isolated peripheral blood mononuclear cells from SLE patients. He found that the levels of Bcl-2 was increased compared with healthy controls. Another study reported slightly decreased Bcl-2 levels in SLE T cells and significantly increased Bcl-2 levels in SLE B cells compared to healthy controls (Kumagai et al., 1993).

Our mRNA findings for *fas* and *bcl-2* expression in lymphocytes under influence of long-term exposure to E2 are comparable with changes seen in some autoimmune diseases. It appears to show that E2 mediates *fas* & *bcl-2* mRNA expression and drives the inappropriate expression of these genes associated with autoimmune diseases. This study also suggests that *fas* and *bcl-2* mRNA expression in thymocytes and splenic T cells is more sensitive than in splenocytes to E2.

Results from our studies (as far as we know) on T cell and B cell, we predict that the network of T and B cell under influence of estrogen show the following: (1). Overdose or imbalance in estrogen, acts on the thymus by preventing negative selection. This negative selection is probably induced by either decreasing *fas* or increasing *bcl-2* expression. (2) Alternatively, E2 may generate mutation in T cell receptors, which results in T cell evasion of negative selection. (3). Over expression of IL-2 may overcome peripheral tolerance in self-reactive T cells present in the microenvironment (Abbas et al., 1994). It has been shown that tolerance of splenocytes from transgenic mice expressing an allogenic MHC class I on β islet cells was reversible *in vitro* by providing exogenous IL-2 to the lymphocytes (Morahan et al., 1989). (4). The increases in lymphokine IFN- γ expression in lymphocytes from by E2-treated mice may be produced by activating an IFN- γ promoter (Fox et al., 1991). (5). Estrogen skews the predominant response from T_{H2} to T_{H1} type resulting in imbalance of lymphokine profiles.

Relative hyperfunction of T_H1 type cells produce IL-2 and IFN- γ , which are involved in inflammatory responses, and in inflammatory cell organ infiltration. Therefore, the increased IL-2 and IFN- γ mRNA expression may be the source of inflammatory response seen in autoimmune diseases. (6). Even through IL-4 mRNA expression is not increased by E2 treatment, the function of T_H2 subset may still be increased, since serological examination shows enhanced levels of antibodies and autoantibodies under E2 influence. There could be other lymphokines not tested in these studies, which may be involved in increased B cell functions. IL-2 also functions on B cells. (7). TGF- β has been shown to promote the switch to IgG2b, which involved in autoimmune diseases (McIntyre et al., 1993).

Considering clinical observations and the effect of E2 on *fas* and *bcl-2* in different lymphocytes, Fas decrease may play a predominant role in apoptotic process of thymocyte and T cell modulation. The main effect is a defect of negative selection in the thymus and hindered apoptosis in periphery processes which normally eliminate self-reactive T cells. Bcl-2 may play a predominant role in splenocyte in regulating B cell function, which produces hypergammaglobulinaemia. There is evidence to show that transgenic MRL/lpr/lpr mice with Fas in T cells, the IgG2a and IgG antibody levels in these mice were decreased to normal, and clinical signs were eliminated. While B cell expression of transgenic *bcl-2* resulted in a higher incidence of autoimmune diseases such as SLE within one year (Strasser et al., 1991, Rose et al., 1994). E2 induces mRNA expression of *fas* and *bcl-2*, that acts on lymphocytes discriminatively. E2 regulation of apoptosis in all lymphocytes is probably achieved by a balance between inducers and inhibitors of apoptosis.

In summary, 17 β -estradiol induces both of T_H1 and T_H2 type responses, which may contribute in autoimmune diseases. In our experiment most of the lymphokines induced by T_H2 subset have not been tested, so we can not conclude that T_H2 cell type was not affected by E2. Furthermore, E2 potentially influences immune response by quantitative and functional means. The inhibitory effect of 17 β -estradiol on lymphocytes appears to be specific for proliferation and was not due to cytotoxicity, Since physiological function of lymphocytes was not damaged by E2 treatment. This suggests that activated T_H1 cells would still carry out their effector functions through the production of lymphokines, but would not expand in number.

CHAPTER 5

GENERAL DISCUSSION

This study is a preliminary investigation of the effects of 17 β -estradiol (E2) on the T-cell mediated immune responses. We focused on the effect of long-term exposure to E2 on lymphokine production and proto-oncogene mRNA expression in C57BL/6J orchietomized inbred male mice. More than 70 different cytokines and many apoptosis-associated gene products have been found to be involved in immune response. We selected lymphokines which belong to T_H1 and T_H2 subsets of T_H population as well as bcl-2 & fas proto-oncogenes to elucidate the relationship between E2 and autoimmune diseases, and to understand the mechanism of E2 modulating immune system.

EFFECT OF E2 ON T CELL SYSTEM

Estrogen has a strong thymolytic effect and also affects several T lymphocyte functions which consequently modulates T cell populations and cell-mediated immune responses. Thymic atrophy can be induced by administration of estrogen, mainly due to a loss of thymocytes (Grossman, 1984). Estradiol treatment in rats results in thymic involution regardless of whether the thymus is of normal size or enlarged as a result of castration. Histological examination demonstrated destruction of thymic lymphocytes and atrophy of thymic lobules (Grossman 1984). Other study has shown that estrogen targets double positive (DP) T cells during T cell differentiation and maturation in the thymus and thus causes the DP subset to decrease. The ratio of CD4⁺ CD8⁻ to CD4⁺ CD8⁺ T cell subset was increased, and the thymocyte numbers were depleted after short-term estrogen treatment in C57BL/6J mice (Screpanti et al., 1989. Silverstone et al., 1994). In earlier studies, it was reported that estrogen increased the numbers of mature thymocytes, reduced circulation CD8⁺ thymic T cells, caused a relative increase in T_H cells, and decreased T_S cells as analyzed by flow cytometry (Novotny et al., 1983; Ansar Ahmed et al., 1985a). Estrogen also enhance the activation of mature B cells leading to higher immunoglobulin production (Grossman 1984).

In recent years, there have been several reports demonstrating the effect of sex hormones (estrogens, progesterone and testosterone) on cytokine expression. In multiple sclerosis patients, 17 β -estradiol increased the production of both IFN- γ and IL-10, and had a biphasic effect on TNF- $\alpha\beta$. In same experimental system, estradiol had no effect on the secretion of IL-4 and TGF- β (Gilmore et al., 1997). Fox et al, (1991) isolated un-separated spleen cells from CD-1 male, followed by treatment with estrogen and incubation with Con-A. They found that short-term exposure to estradiol increased IFN- γ mRNA expression in Con-A treated murine spleen cells. Viselli et al. (1995) studied the effects of androgen deficiency in normal male C57BL/6J mice. They found that production of IL-2 and IFN- γ by ConA stimulated un-separated spleen cells was increased in cells from castrated males versus cells from intact males.

RESULTS

The findings in our study demonstrated that E2-treated mice exhibited reduced viability of thymocytes, T cells and splenocytes compared with PO-treated mice. Of these three cell types, thymocytes and splenic enriched-T cells were more sensitive to E2 treatment than mixed splenic lymphocytes. E2 also selectively influenced lymphokine mRNA expression. Treatment increased the production of IL-2, IFN- γ , and TGF- β_1 , but did not affect IL-4 production. Our findings

regarding lymphokines IL-2, IFN- γ , and IL-4 were in agreement with previous research (Fox et al, 1991, Viselli et al., 1995, Gilmore et al., 1997). However, our results for TGF- β_1 differed from previous finding, possibly because of different treatment regiment and mouse model used.

Treatment with E2 also affected *fas* and *bcl-2* proto-oncogene expression. The profiles showed *fas* mRNA to be decreased, while *bcl-2* mRNA increased. Our findings regarding the effects of long-term of exposure to E2 on proto-oncogene *fas* and *bcl-2* mRNA expression in thymocytes, T cells, and splenocytes is, to our knowledge, the first report in normal mice (i.e. without autoimmune diseases).

SIGNIFICANCE

Estrogen's role in the regulation of immune responses has long been recognized. However, the regulatory mechanism and underlying relationship between autoimmune diseases and estrogen have not been fully elucidated. In recent years, several reports indicated that estrogen hyperactivates B cells, causing a relative increase of the activity of B cell population (Ansar Ahmed, personal communication), and increases in antibodies and autoantibodies (Ansar Ahmed et al., 1990, 1993a, 1993b, Verthelyi et al, 1994, Roubinian et al., 1978, Carlsten et al., 1990). It is known that T cells play a major role in immune responses and that B cell function depends on lymphokines secreted by T cells and direct contracts with T cells. Our study explored some of the effects of estrogen on T cell function . We found that estrogen mediates immune responses primarily by influencing lymphokine production. This influence on lymphokine production regulates the function of T cells, B cells, NK cells, macrophages, and epithelial cells. Additionally estrogen increases the ratio of T_H1 and T_H2 cell type responses, mainly by affecting the T_H1 cell type, which deranges immune function and may be a critical feature in initiating autoimmune diseases. The balance and regulation of T_H1 and T_H2 populations may have therapeutic implications for autoimmune diseases management. Further, the immune system may be regulated by estrogen through alteration in apoptosis of lymphocytes.

FURTHER WORK NEED TO BE DONE

Unfortunately, this study was unable to address all of the effects of estrogen on T-cell mediated immunity (T-CMI). Additional studies may address: effects of estrogen on other lymphokines, in order to establish a more complete picture about the effects of estrogen on T-CMI; lymphokines produced by the CD4⁺ CD8⁻ T cell population; and utility of competitive RT-PCR for quantitation of mRNA. Production of lymphokines in cell supernatants should be evaluated with simultaneous investigation of lymphokine mRNA expression; IL-2, and IL-4 cell surface receptors need also be investigated.

In the Alamar Blue study, we tested the functional utilization of lymphocytes following a non-radioactive lymphocyte proliferation assay (Lympho-Pro assay or Alamar Blue assay) including: mRNA, apoptosis, cytogenetic, and immunophenotyping analysis in normal lymphocytes. In most clinical and experimental investigations, it may be necessary to perform many tests on lymphocytes collected from individuals with various pathological conditions, such as autoimmune diseases or cancer. Since available and survival lymphocytes were limited after E2 treatment, we did additional experiments to evaluate if the E2 treated lymphocytes could be utilized after undergoing Lympho-Pro assay. Alamar Blue dye did not interfere with β -actin, IL-2, IL-4, IFN- γ , and TGF- β mRNA expression of splenocyte. Cell cycle analysis yielded

inconsistent results from two experiments. Apoptosis analysis was done only once (data were not shown). Therefore, further study of E2-treated cells which have been subjected to the Alamar Blue assay is necessary in order to clarify if biological activation of E2 treatment cells are interfered by Alamar Blue.

Table 4.1. Serum levels of estrogen in C57BL/6J mice *

Strain	Sex	Treatment	Serum conc. of E2
C57BL/6J	Female	NO	345.7 ± 152.1 pg/ml
	Male	NO	67.9 ± 5.8 pg/ml
	Male	orx and E2 implant	425.3 ± 29.1 pg/ml
	Male	sham orx/sham implant	82.3 ± 18.8 pg/ml
	Male	orx/sham implant	66.8 ± 5.2 pg/ml

* Ansar Ahmed et al., 1989.

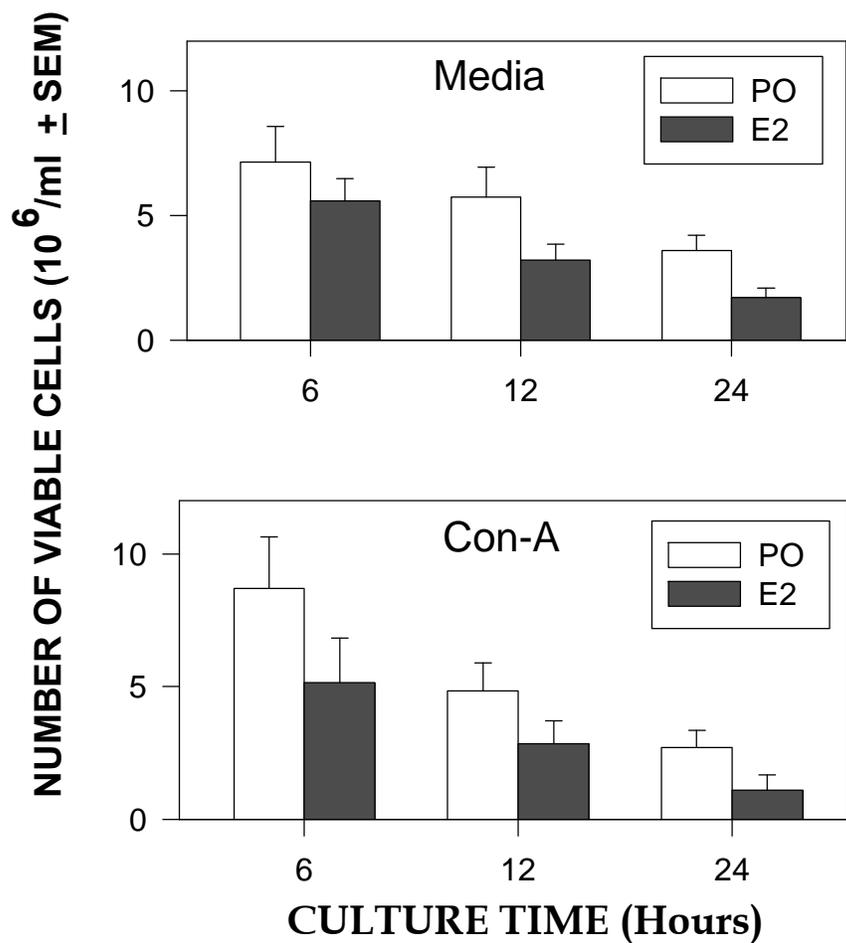


Figure 4.3A. Viability of thymocytes isolated from PO or E2 treated mice and cultured at 5×10^5 cells/well. Thymocyte viability was determined by trypan blue exclusion assay at 0, 6, 12, and 24 hours after cultivation with media alone or media with Con-A ($10\mu\text{g/ml}$) stimulation.

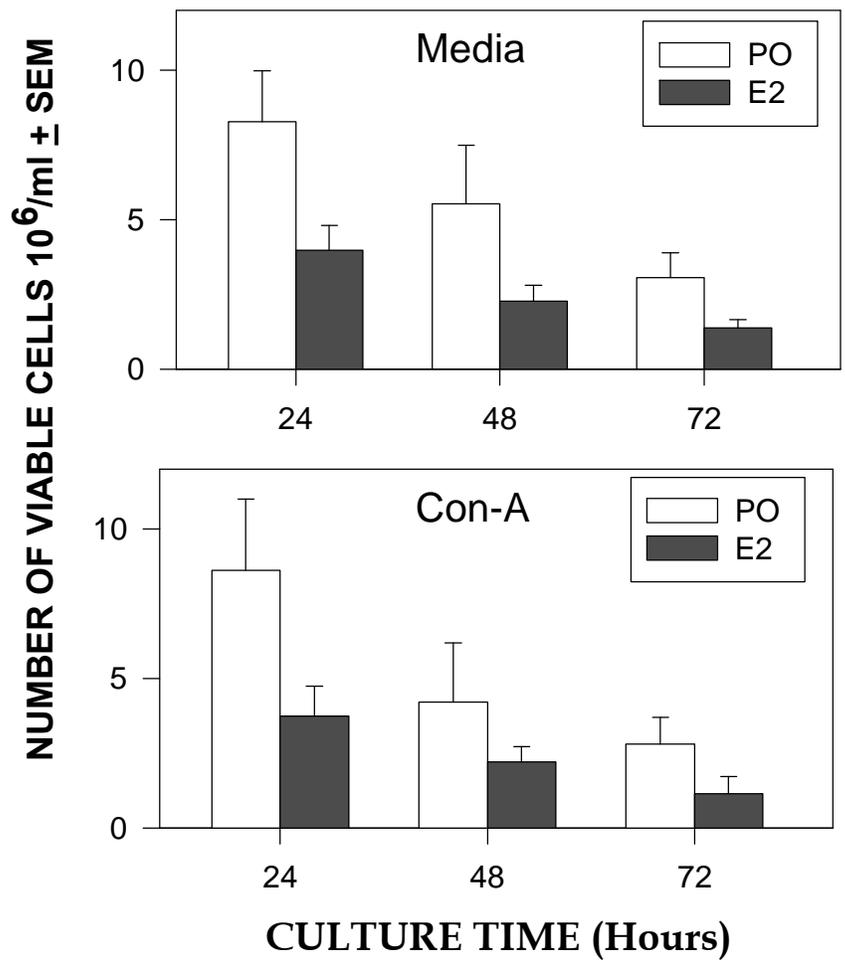


Figure 4.3B. Viability of enriched splenic T cells isolated from PO or E2 treated mice and cultured at 5×10^5 cells/well. T cell viability was determined by trypan blue exclusion assay at 0, 24, 48, and 72 hours after cultivation with media alone or media with Con-A ($10\mu\text{g/ml}$) stimulation.

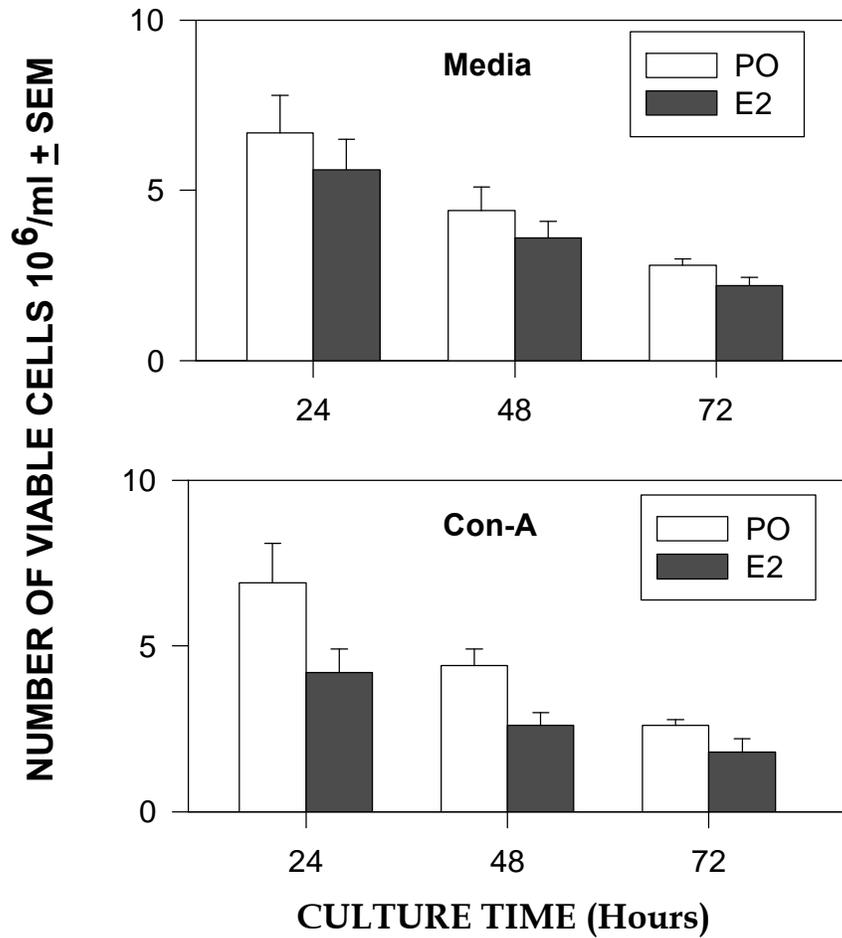


Figure 4.3C. Viability of splenic un-separated lymphocytes isolated from PO or E2 treated mice and cultured at 5×10^5 cells/well. Splenocyte viability was determined by trypan blue exclusion assay at 0, 24, 48, and 72 hours after cultivation with media alone or media with Con-A ($10\mu\text{g/ml}$) stimulation.

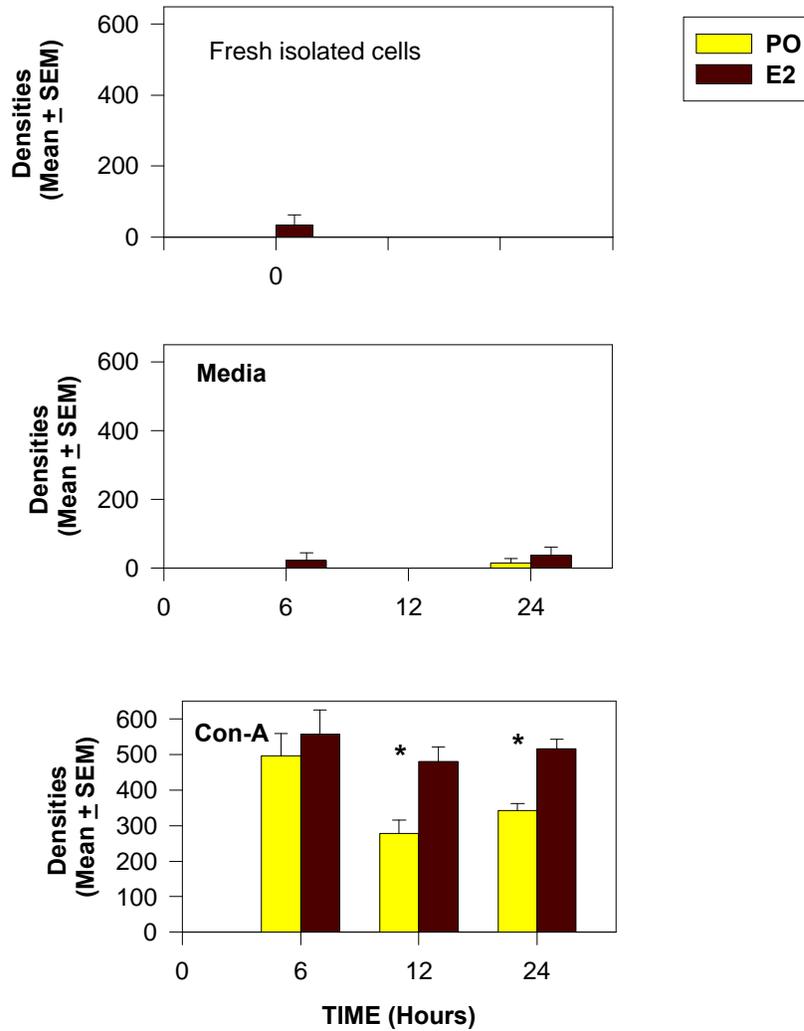


Figure 4.4. IL-2 mRNA expression in thymocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-6 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulation for 6, 12, and 24 hours. RNA was isolated from thymocytes at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry of electrophoretograms.

Note: * P < 0.05

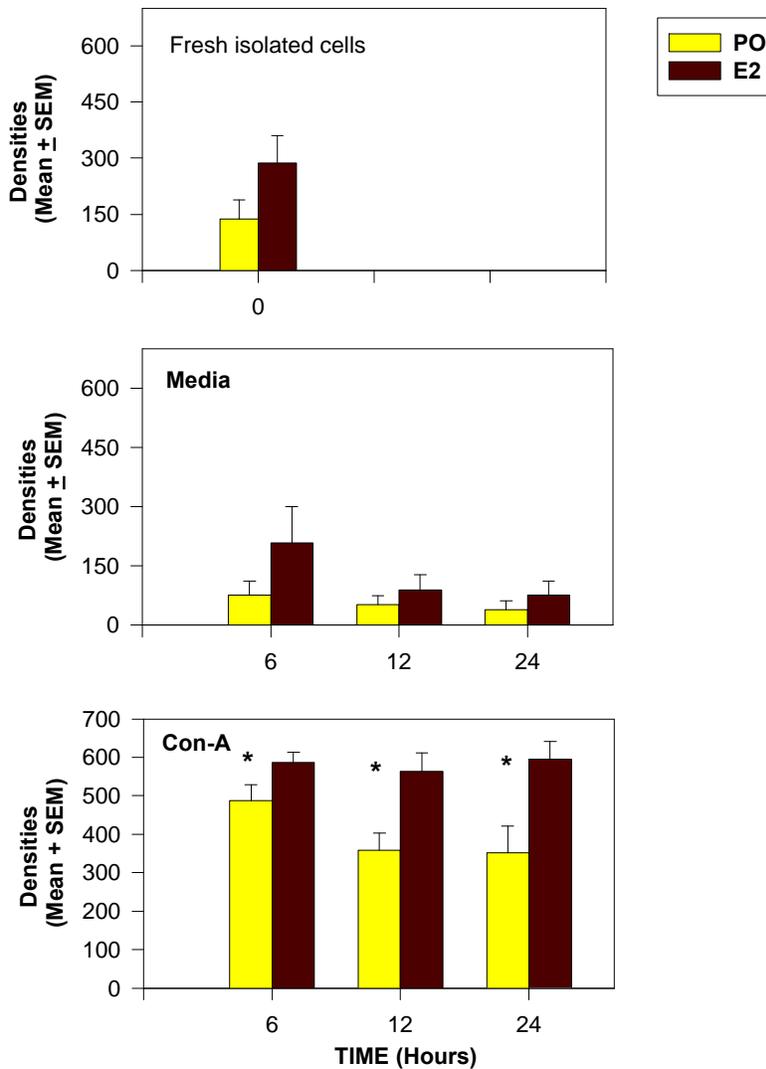


Figure 4.5. IFN- γ mRNA expression in thymocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-6 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulation for 6, 12, and 24 hours. RNA was isolated from thymocytes at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry of electrophoretograms.

Note: * $P < 0.05$

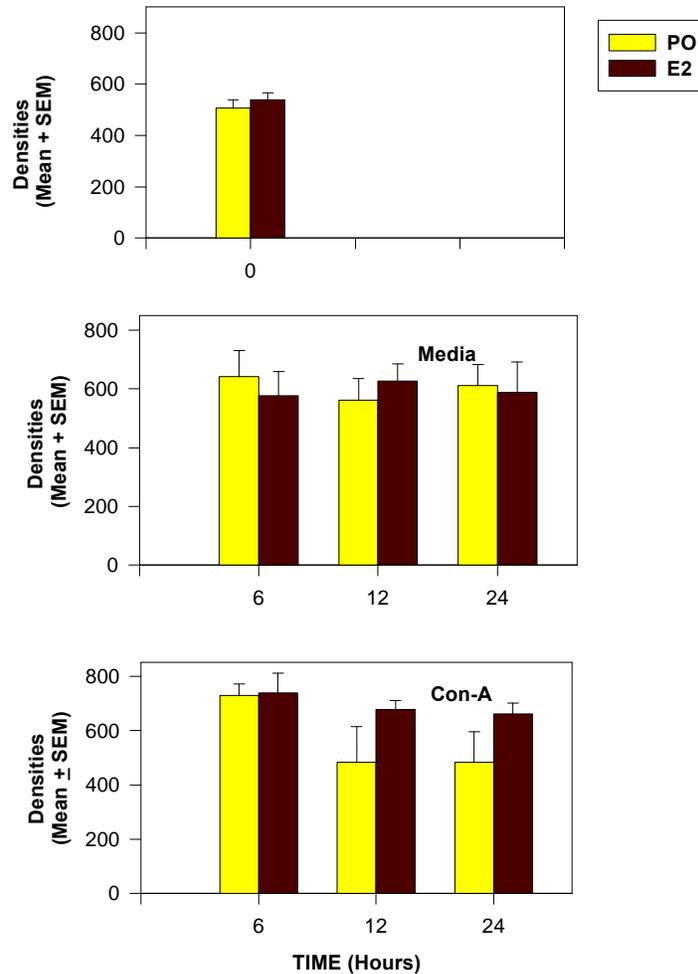


Figure 4.6. TGF- β mRNA expression in thymocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-6 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulation for 6, 12, and 24 hours. RNA was isolated from thymocytes at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry electrophoretograms.

Note: * $P < 0.05$

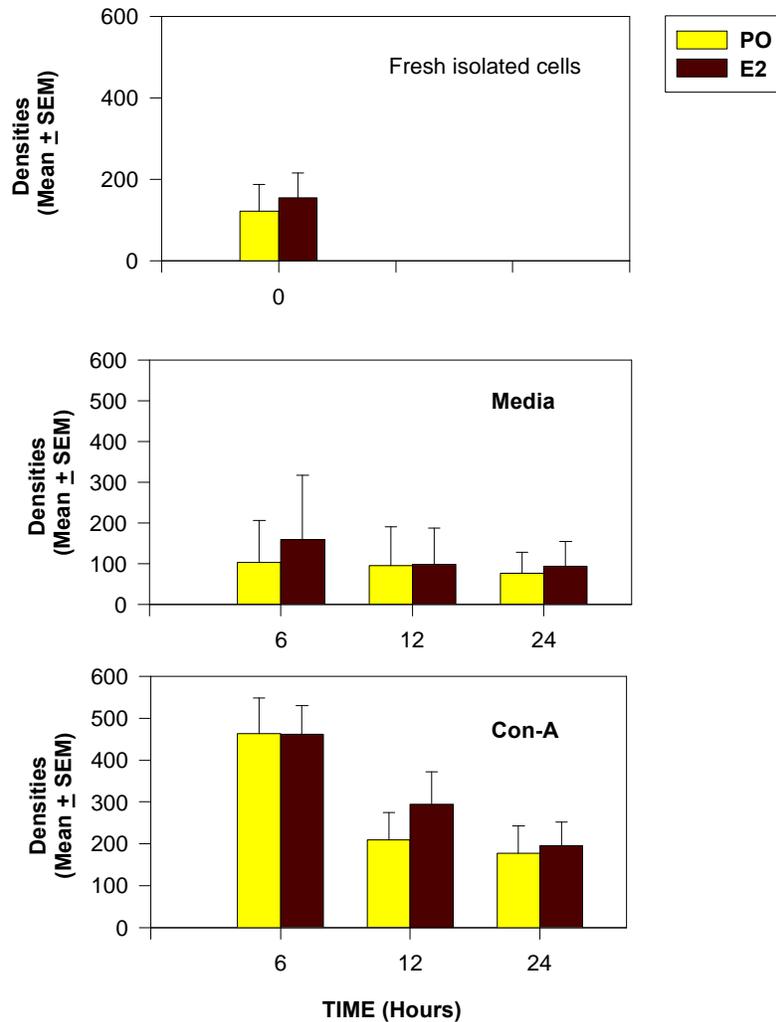


Figure 4.7. IL-4 mRNA expression in thymocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-6 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulation for 6, 12, and 24 hours. RNA was isolated from thymocytes at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry of electrophoretograms.

Note: * $P < 0.05$

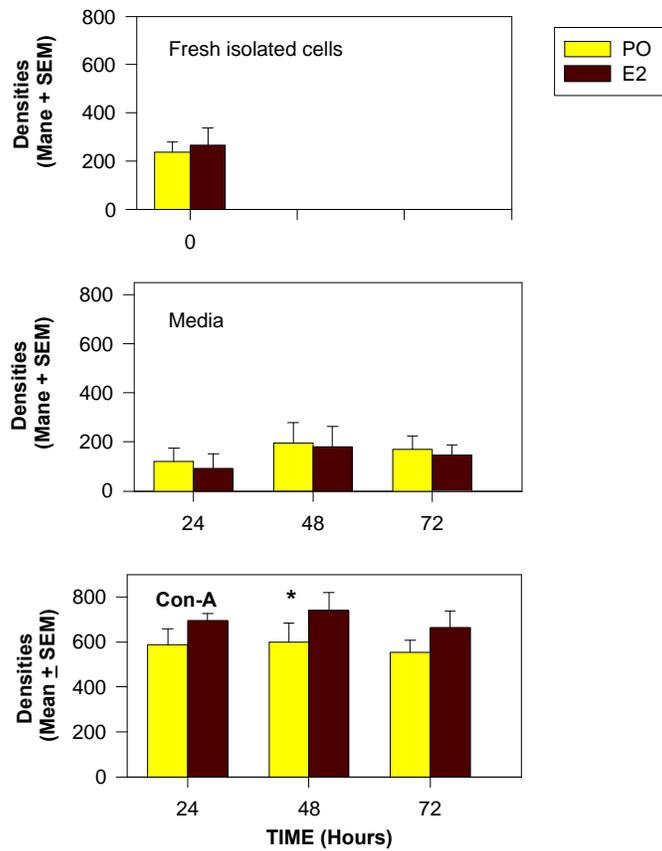


Figure 4.8. IL-2 mRNA expression in enriched splenic T cells of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-6 months. T cells were purified from splenocytes and cultured with media alone or media with mitogen Con-A stimulation for 24, 48, and 72 hours. RNA was isolated from T cells at different culture times, subjected to RT-PCR. PCR products were quantified using densitometry of electrophoreograms.

Note: * $P < 0.05$

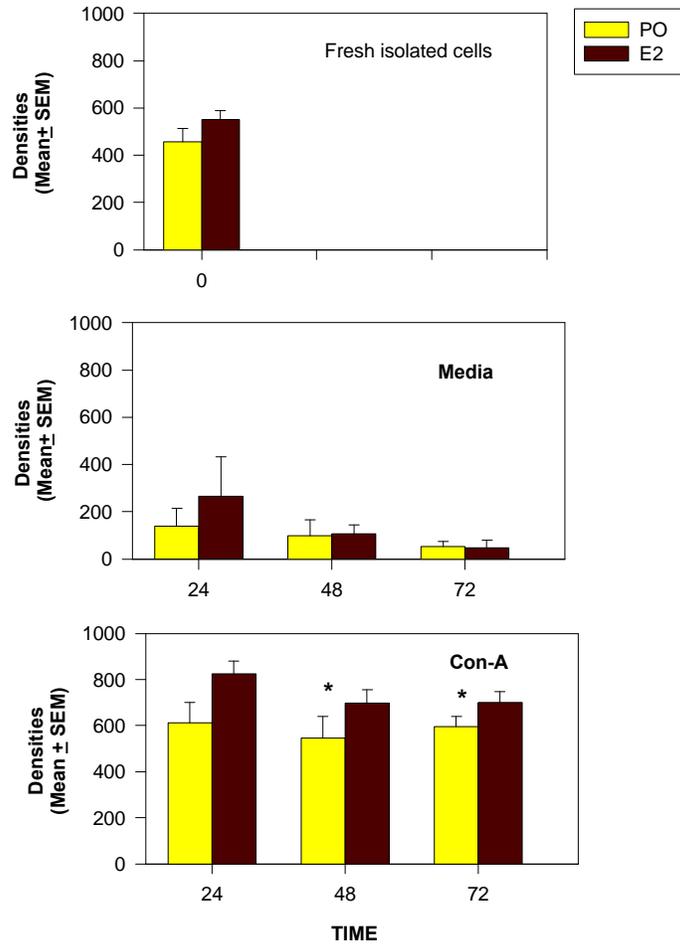


Figure 4.9. IFN- γ mRNA expression in enriched splenic T cells of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. T cells were purified from splenocytes and cultured with media alone or media with mitogen Con-A stimulation for 24, 48, and 72 hours. RNA was isolated from T cells at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry of electrophoretograms.

Note: * $P < 0.05$

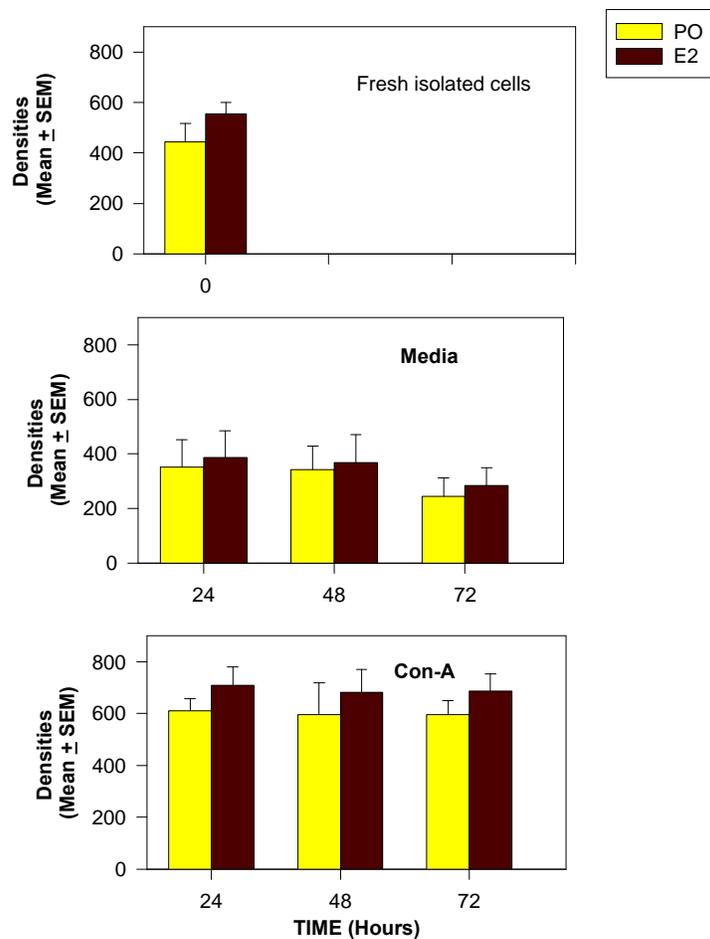


Figure 4.10. TGF- β mRNA expression in enriched splenic T cells of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. T cells were purified from splenocytes and cultured with media alone or media with mitogen Con-A stimulation for 24, 48, and 72 hours. RNA was isolated from T cells at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry of electrophoreograms.

Note: * P < 0.05

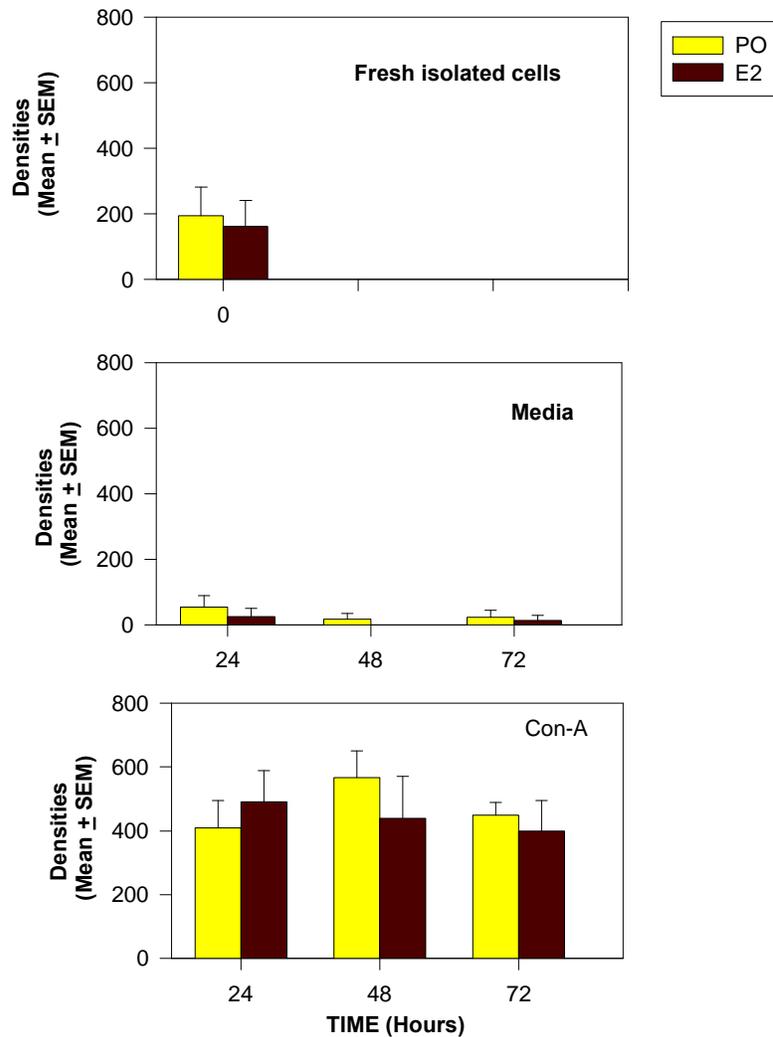


Figure 4.11. IL-4 mRNA expression in enriched splenic T cells of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. T cells were purified from splenocytes and cultured with media alone or media with mitogen Con-A stimulation for 24, 48, and 72 hours. RNA was isolated from T cells at different culture times, and subjected to RT-PCR. PCR products were quantified using densitometry of electrophoretograms.

Note: * $P < 0.05$

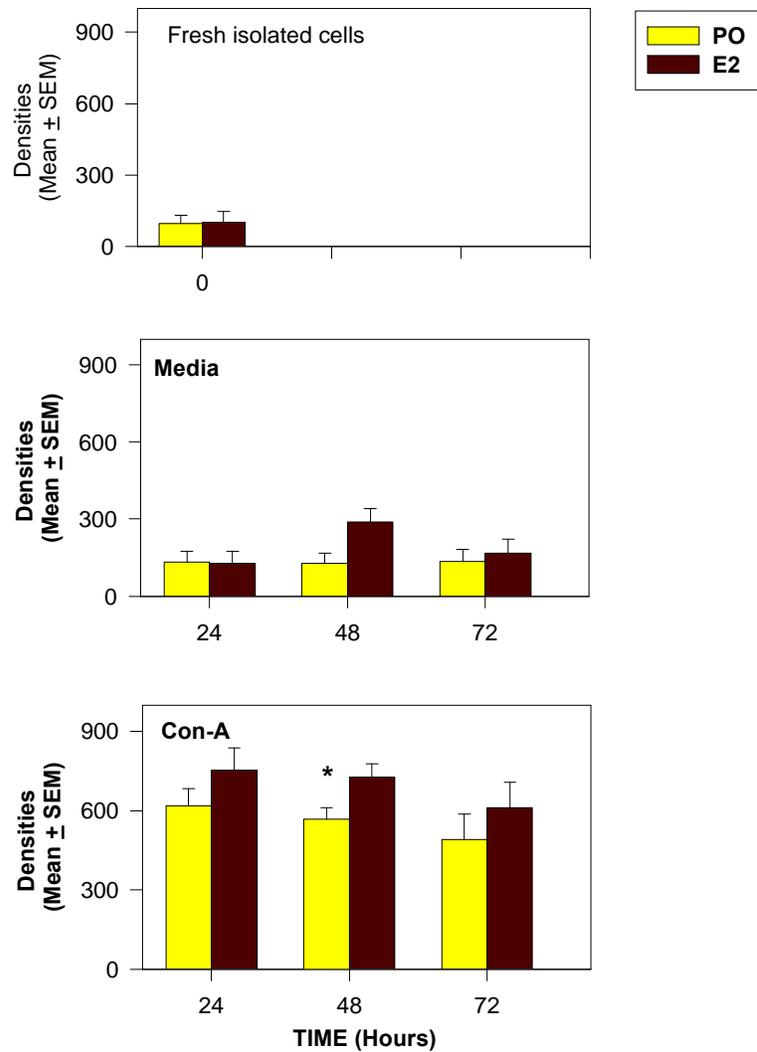


Figure 4.12. IL-2 mRNA expression in splenocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from splenocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * P < 0.05

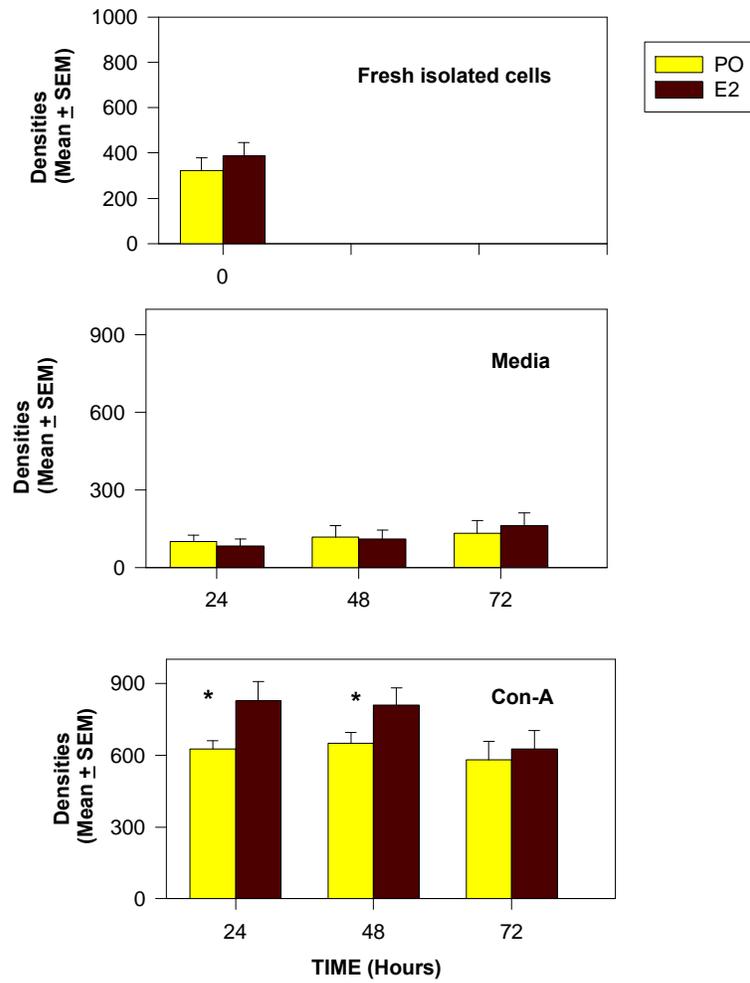


Figure 4.13 IFN- γ mRNA expression in splenocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from splenocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

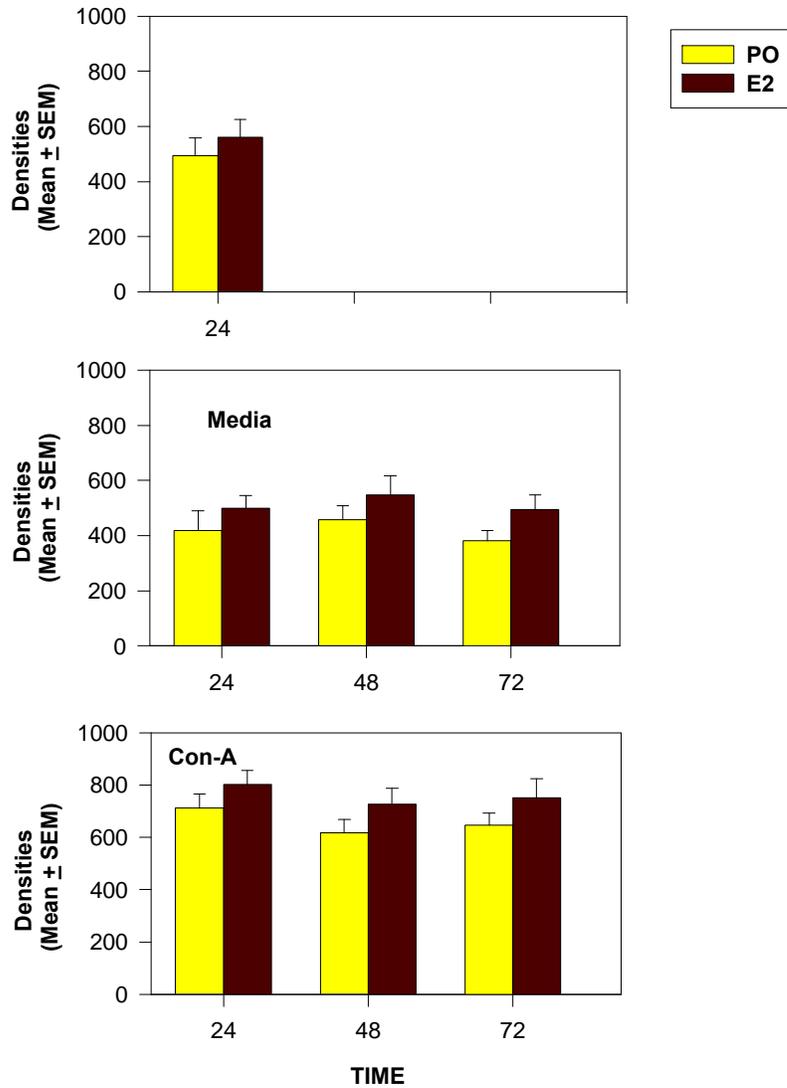


Figure 4.14 TGF- β mRNA expression in splenocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from splenocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * P < 0.05

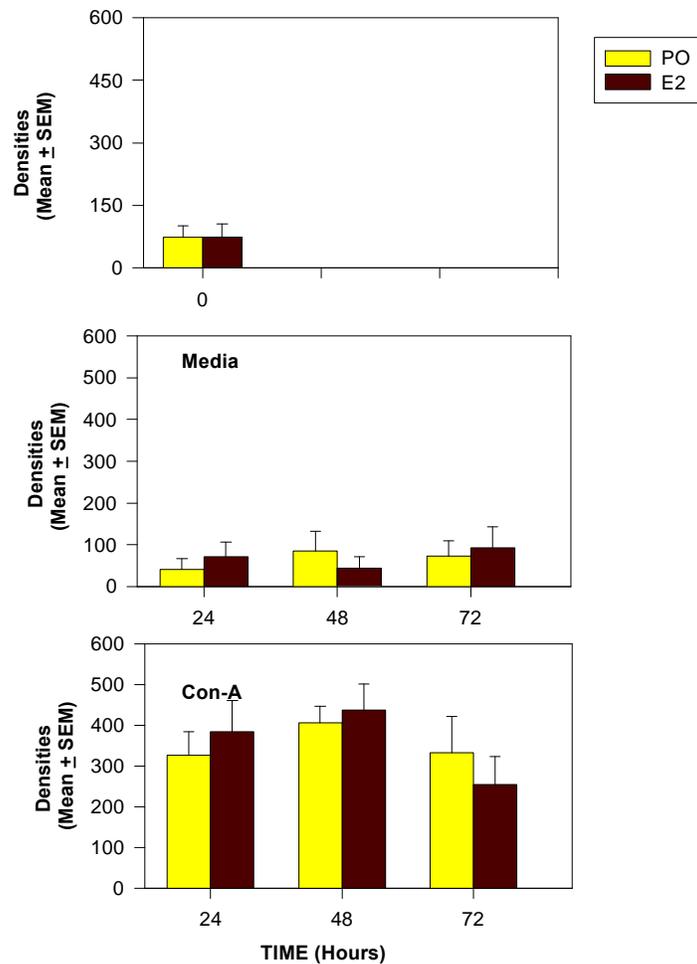


Figure 4.15 IL-4 mRNA expression in splenocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from splenocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

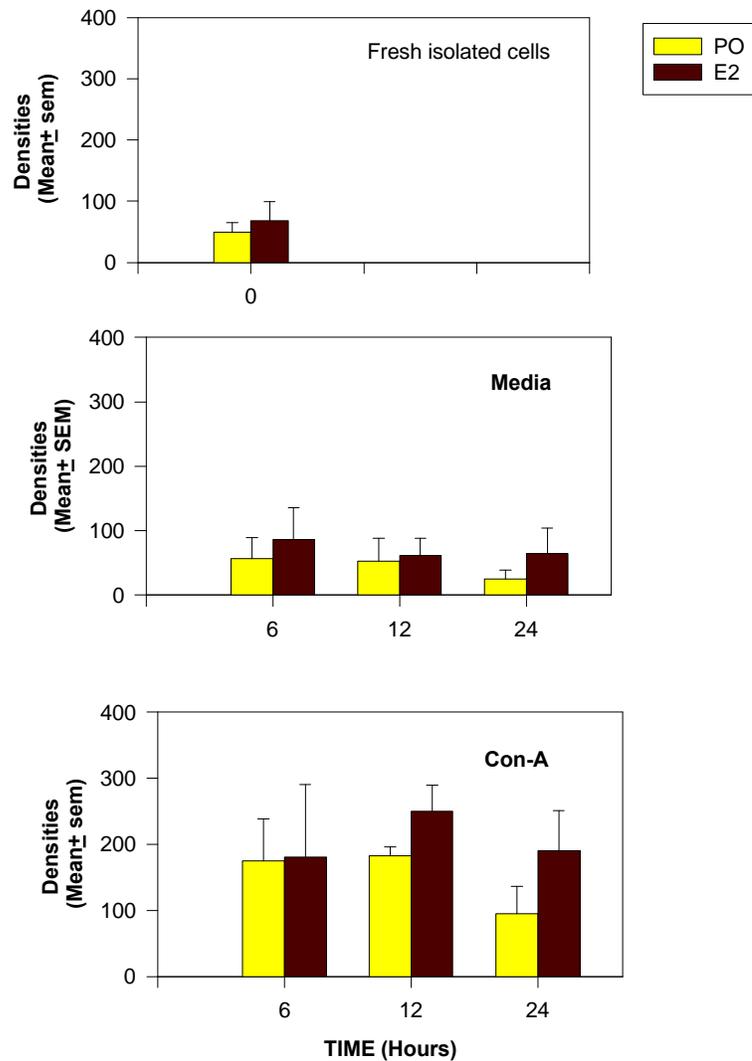


Figure 4.16 *bcl-2* proto-oncogene mRNA expression in thymocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 6, 12, and 24 hours. RNA was extracted from thymocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

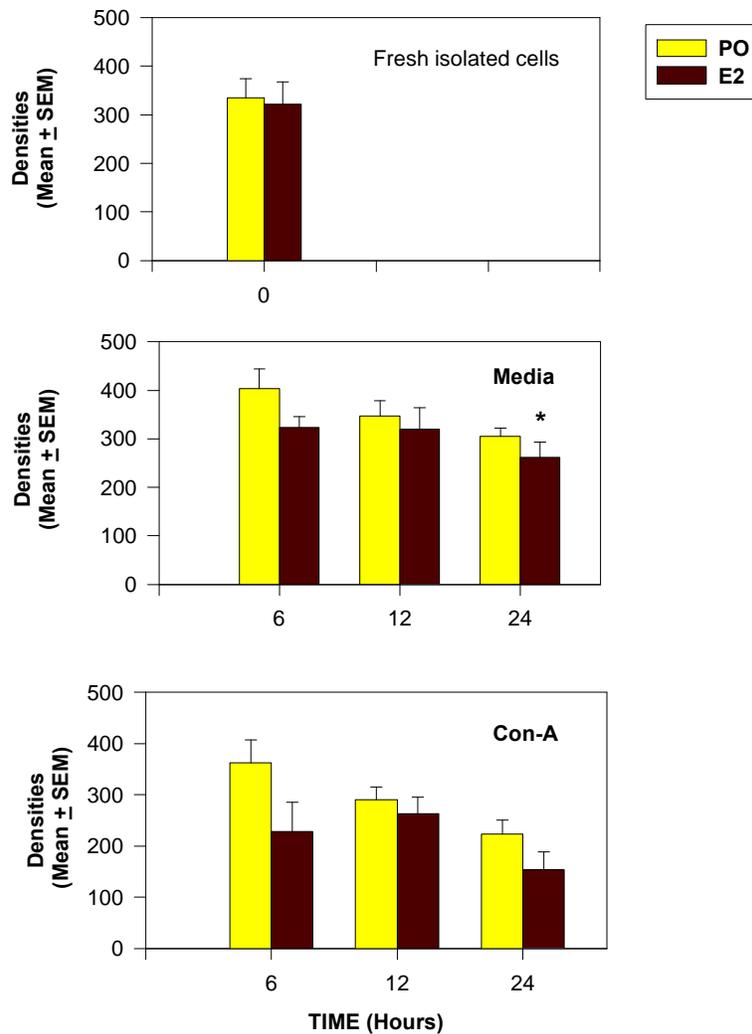


Figure 4.17 *fas* proto-oncogene mRNA expression in thymocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 6, 12, and 24 hours. RNA was extracted from thymocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

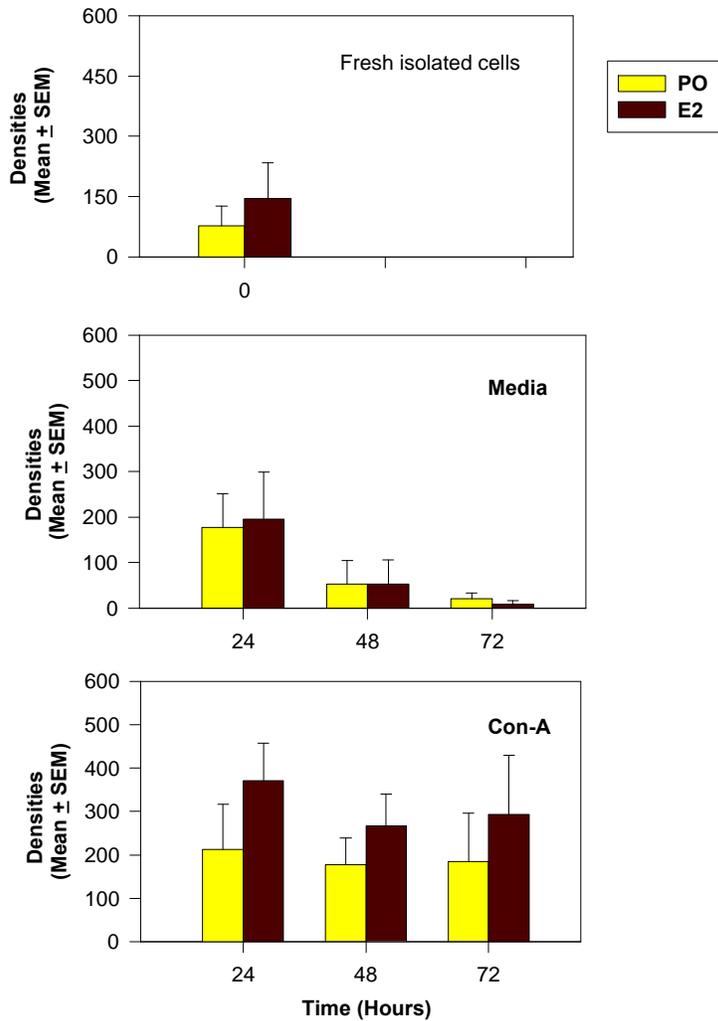


Figure 4.18. *bcl-2* proto-oncogene mRNA expression in enriched splenic T cells of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. T cells were purified from splenocytes and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from T cell at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * P < 0.05

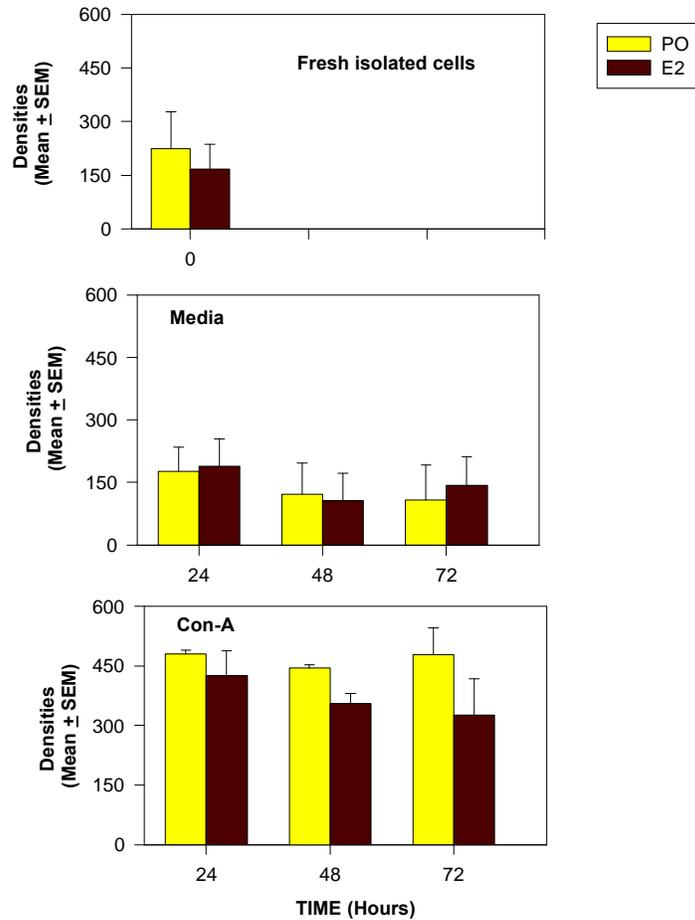


Figure 4.19. *fas* proto-oncogene mRNA expression in enriched splenic T cells of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. T cells were purified from splenocytes and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from T cell at different culture times followed by RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

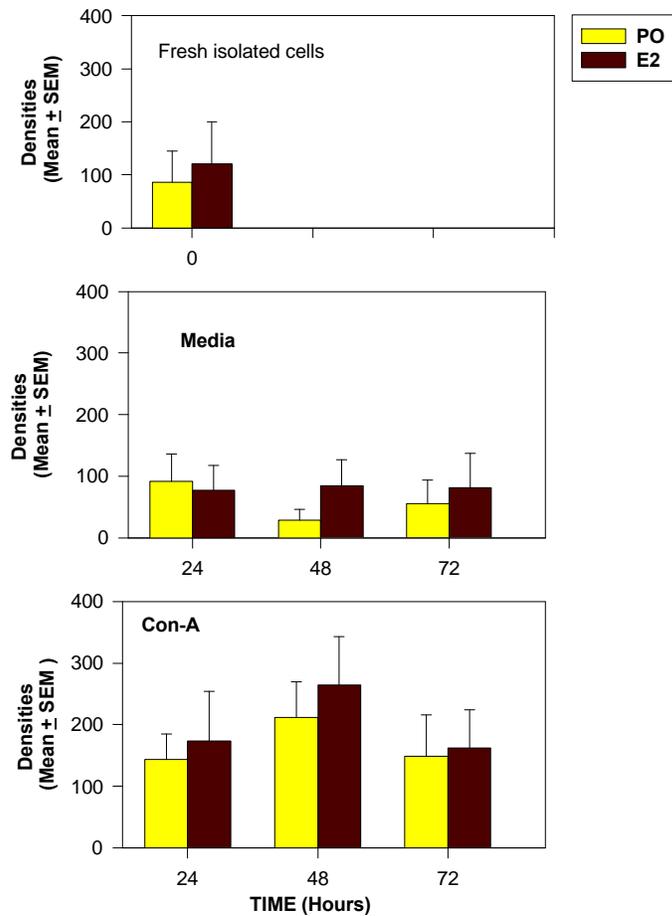


Figure 4.20. *bcl-2* proto-oncogene mRNA expression in splenocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from splenocytes at different culture times followed by RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

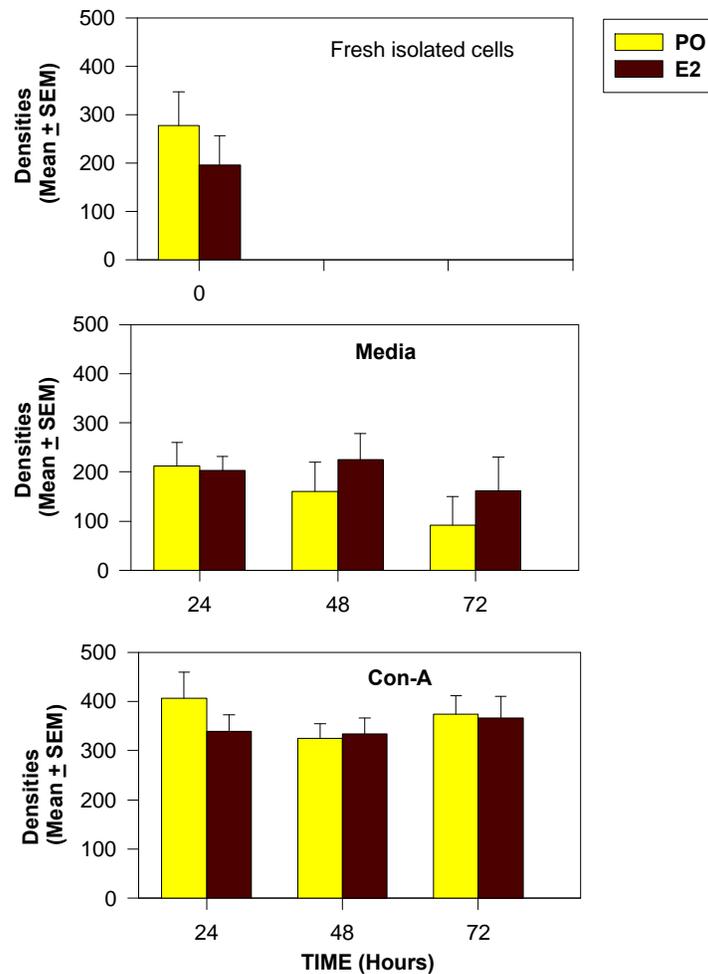


Figure 4.21. *fas* proto-oncogene mRNA expression in splenocytes of C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from splenocytes at different culture times and subjected to RT-PCR. The PCR products were quantified by densitometer.

Note: * $P < 0.05$

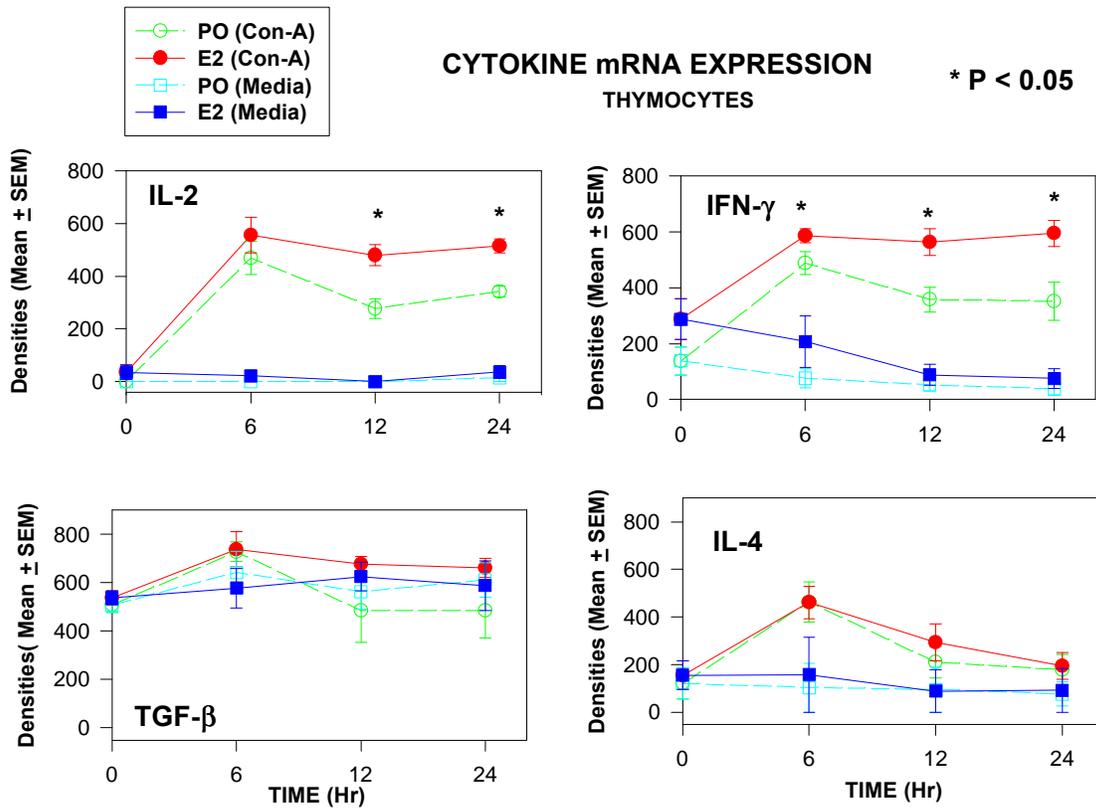


Figure 4. 22 Overall effects of E2 on lymphokine mRNA expression of thymocytes in C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Thymocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 6, 12, and 48 hours. RNA was extracted from thymocytes at different culture times and subjected to RT-PCR. The PCR products, IL-2, IFN- γ , TGF- β , and IL-4, were quantified by densitometer.

Note: * P < 0.05

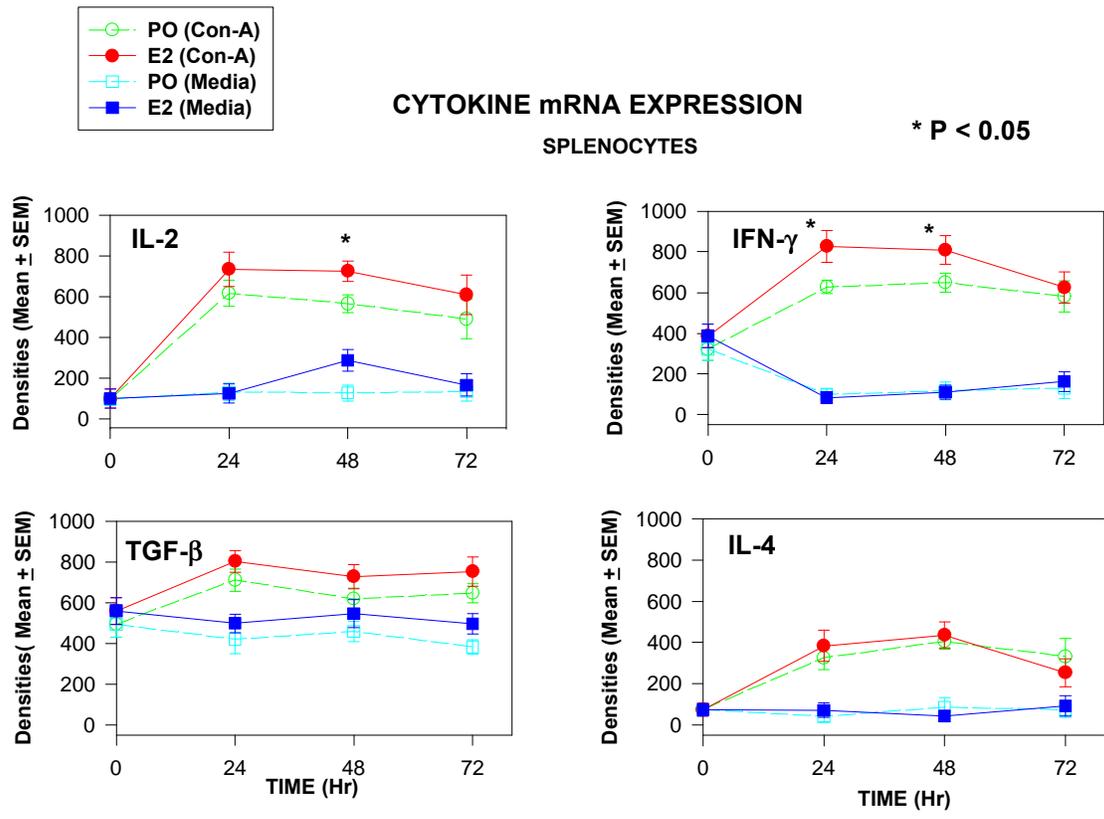


Figure 4. 23 Overall effects of E2 on lymphokine mRNA expression of splenic lymphocytes in C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. Splenic lymphocytes were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from T cells at different culture times and subjected to RT-PCR. The PCR products, IL-2, IFN- γ , TGF- β , and IL-4, were quantified by densitometer.

Note: * P < 0.05

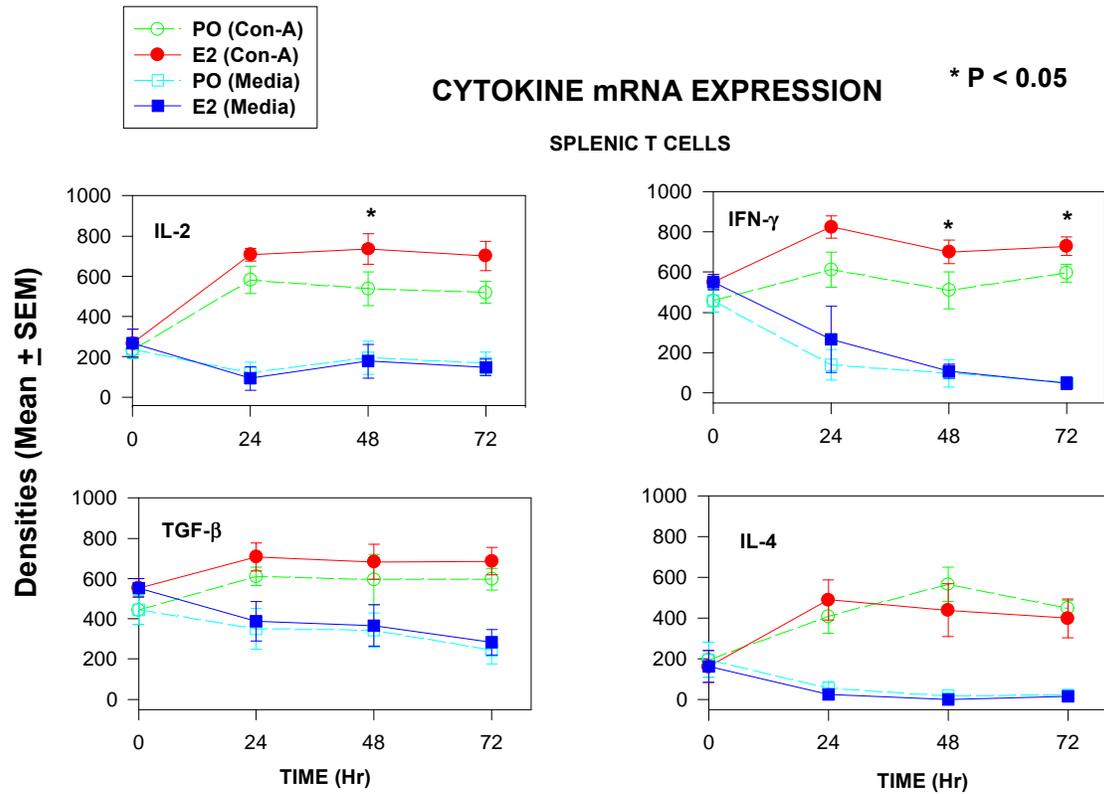


Figure 4. 24 Overall effects of E2 on lymphokine mRNA expression of enriched splenic T cells in C57BL/6J PO and E2 treated mice. C57BL/6J mice were treated with PO or E2 for 3-4 months. T cells were isolated and cultured with media alone or media with mitogen Con-A stimulated for 24, 48, and 72 hours. RNA was extracted from T cells at different culture times and subjected to RT-PCR. The PCR products, IL-2, IFN- γ , TGF- β , and IL-4, were quantified by densitometer.

Note: * P < 0.05

REFERENCES

- Abbas, A. K., Lichtman, A.H. , and Pober, J. S. 1994. Cellular and Molecular Immunology. W. B. Saunders Company.
- Adachi, K., Kumamoto, T. and Araki, S. 1989. Interleukin-2 receptor levels indicating relapse in multiple sclerosis. *Lancet* (Jan-Mar): 559-560
- Ahn, Y. S., Harrington, W. J., Simon, S. R., Mylvaganam, R., Pall, L. M. and So, A. G. 1983. Danazol for the treatment of idiopathic thrombocytopenic purpura. *N. Engl. J. Med.* 308:1396.
- Amino, N., Miyai, K. , Yamamoto, K. 1977 b. Transient recurrence of hypothyroidism after delivery in Grave's diseases. *J. Clin. Endocrinol. Metab.* 44:130-136.
- Amino, N., Miyai, K. , Kuro, R., Tanizawa, O. , Azukizawa, M. , Takai, S. , Tanaka, E., Nishi, K., Kahuashima, M., and Kumahara, Y. 1977 a. Transient postpartum hypothyroidism: fourteen cases with autoimmune thyroiditis. *Ann Int. Med.* 87:155-159.
- Anderson, J.T., Cornelius, J.G., Jerpe, A.J., Winter, W.E. , and Peck, A.B. 1993. Insulin-dependent diabetes in the NOD mouse model. II. Beta cell destruction in autoimmune diabetes is TH2 and not TH1 mediated event. *Autoimmunity.* 15:113-122.
- Ansar Ahmed, S., Michael, D., and Norman, T. 1985b. Effects of short-term administration of sex hormone on normal and autoimmune mice. *J. Immunol.*134:204-210.
- Ansar Ahmed, S., and Penhale, W. J. 1980. The role of sex steroids in autoimmune thyroiditis. Fourth international congress of immunology. Paris Abstract. Edited by JL Preud' Homme and VAL Hawken. Paris. Academic press, p 14.3.02.
- Ansar Ahmed, S., Dauphinee, M.J., and Talal, N. 1985a. Effects of short term administration of sex hormones on normal and autoimmune mice. *J. Immunol.* 134 (1): 204-210.
- Ansar Ahmed, S., Dauphinee, M.J., Montoya, A.L., and Talal, N. 1989. Estrogen induced normal murine CD5+ B cells to produce autoantibodies. *J. Immunol.* 142: 2647-2653.
- Ansar Ahmed, S., Talal, N., and Christadoss, P. 1987. Genetic regulation of testosterone induced immune suppression. *Cell. Immunol.* 104: 91-98.
- Ansar Ahmend, S., Young, P.R, and Penhale, W.J. 1986. Beneficial effects of testosterone in the treatment of chronic autoimmune thyroiditis in rats. *J. Immunol.* 136: 143-147.
- Ansar Ahmend, S., Penhale, W. J., and Talal, N. 1985. Sex hormones, immune responses, and autoimmune diseases. Mechanism of sex hormone action. *Am. J. Pathol.* 121: 531-551.
- Ansar Ahmend, S., and Talal, N. 1988. Sex steroid, sexsteroid receptors and autoimmune diseases. In Sheridan PS, Blum K & Trachtengerg MC (eds) *Steroid Receptos and Diseases: Cancer, autoimmune diseases, bone and circulatory disorders.* 289-36. New York:Maecel Dekker.
- Ansar Ahmend, S., and Talal, N. 1990. Sex hormones and the immune system-part 2. *Animal data. Bailliere's Clinical Rheumatology.* 4 (1): 13-31.
- Ansar Ahmend, S., and Verthelyi, D. 1993a. Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but no dihydrotestosterone. *J. Autoimmunity.* 6:265-279.
- Ansar Ahmend, S., Boone, J., and Verthelyi, D. 1993b. Anticardiolipin antibodies in autoimmune-prone BXSb and MRL/LPR mice, and estrogen-treated normal C57BL/6J mice crossreact with other phospholipids. *Int. J. Immunopathol. Pharmacol.* 6 (3): 135-147.
- Ansar Ahmed, S., Gogal, Jr., R. M. and Walsh, J. E. (1994) A new rapid and non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J. Immunol. methods* 170, 211

- Apelgren, L. D., Bailey, D. L., Fouts, R. L., Short, L., Bryan, N., Evans, G. F., Sandusky, G. E., Zuckerman, S. H., Glasebrook, A., and Bumol, T. F. 1996. The effect of a selective estrogen receptor modulator on the progression of spontaneous autoimmune disease in MRL/lpr/lpr mice. *Cell. Immunol.* 173:55-63.
- Araneo B. A., Dowell, T., Diegel, M., and Daynes, R. A. 1991. Dipydrotestosterone exerts a depressive influence on the production of interleukine-4 (IL-4), IL-5, and gamma-interferon, but not IL-2 by activated murin T cells. *Blood* 78:688-699.
- Ariga H., Edwards, J., and Sullivan, D. A. 1989. Androgen control of autoimmune expression in lacrimal gland of MRL-MP/lpr-lpr mice. *Clin.Immun.and immunopath.* 53:499-508.)
- Aust, G., Lehmann, I., Lave, S., and Scherbaum, W.A. 1996. Activated and interferon- γ producing thyroid-derived T cells are detected in Grave s diseases, thyroid autonomy as well as in non-toxic multinodular goiter. *European J.Endocrinol.*135:60-68.
- Ben-Hur, H., Mor, G., Insler, V., Blickstein, I., Amir-Zaltsman, Y., Sharp, A., Globerson, A., and Kohen, F.1995. Menopause is associated with a significant increase in blood monocyte number and relative decrease in the expression of estrogen receptors in human peripheral monocytes. *Am. J. Reprod. Immunol.* 34: 363-369.
- Beato, M. 1989. Gene regulation by steroid hormones. *Cell.* 56 (3):335-344.
- Ben-Chetrit, A., and Ben-Chetrit, B. 1994. Systemic lupus erythematosus induced by ovulation induction treatment. *Arthritis & Rheumatism.* 37:1614-1617.
- Billiau, A. 1996. Interferon- γ . Biology and role in pathogenesis. *Adv. Immunol.* 62: 61-130.
- Birk, K., and Rudick, R. 1986. Pregnancy and multiple sclerosis. *Archives of Neurology.* 43:719-726.
- Birk, K., Ford, C., Smeltzer, S., Ryan, D., Miller, R., and Rudick, R. A. 1990. The clinical course of multiple sclerosis during pregnancy and the puerperium. *Archieives of neurology.* 47:738-742.
- Birner, I. M. 1945. Pregnancy and multiple sclerosis: case report. *New York State Journal of medicine.* 45:634-635.
- Bishop, C.J., Moss, D.J., Ryan, J.M., and Burrows, S.R. 1885. T lymphocytes in infectious mononucleosis. II. Response *in vitro* to interleukin-2 and establishment of T cell lines. *Clin. Exp. Immunol.* 60: 70-77.
- Blackman, M., Kappler, J., and Marrack, P. 1990. The role of the T cell receptor in positive and negative selection of developing T cells. *Science* 248, 1335-1341.
- Boise, L. H., Patricia, J.N., and Craig, B. T. 1995. CD28 and apoptosis. *Current Opinion in immunology* 7: 620-625.
- Carbone, A., Piantelli, M., Musiani, P., Larocca, L. M., Aiello, F. B., Maggiano, N., Scoppetta, C., Crucitti, F., and Ranelletti, F.O. 1986. Estrogen binding sites in peripheral blood mononuclear cells and thymocytes from 2 myasthenia gravis pateints. *J. Clin.Lab. Immunol.* 21:87-91.
- Carlsten, H., Nilsson, N., Jonsson, B.K., Holmdahl, R., Tarkowski, A. 1992. Estrogen accelarates immune complex glomerulonephritis but ameliorates T cell- mediated vasculitis in autoimmune MRL lpl/lpl mice. *Cell Immunol.* 144:190-202.
- Charles, L. S., Shutter, J. R., Hockenbery, D., Kanagawa,O., and Korsmeyer, S. J. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *cell.* 67:879-888.

- Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A., and Weiner, H.L. 1994. Regulatory T cell clone induced by oral tolerance: Suppression of autoimmune encephalitis. *Science*. 265: 1237-1240.
- Cheevers, W.P., Perry, L.L., Wikerson, M.J., and Hullinger, G. A. 1994. Type 2 immune response to viral antigens in lentivirus induced arthritis. *Arth. Rheum.* 37(9-12): S397.
- Chopra, I. J. and Tulchinsky, D. 1974. Status of estrogen-androgen balance in hyperthyroid men with Graves' disease. *J. Clin. Endocrinol. Metab.* 38:269-277.
- Cohen, J.H.M., Danel, L., Cordier, G., Saez, S., and Revillard, J.P. 1983. Sex steroid receptors in peripheral T cells: Absence of androgen receptors and restriction of estrogen receptors to OKT 18-positive cells. *J. Immunol.* 131:2767-2671.
- Cohen, J.J. 1991. Programmed cell death in immune system. *Adv. Immunol.* 50:55-85
- Cohen, J.J., and Richard, C.D. 1992. Apoptosis and programmed cell death in immunity. *Annu. Rew. Immunol.* 10: 267-293
- Cohen, J.J., Duke, R.C., Chervenak, R., Sellins, K.S., and Olson, L.K. 1985. DNA fragmentation in targets of CTL: an example of programmed cell death in the immune system. *Adv. Exp. Med. Biol.* 184:493-506
- Danel, L., Vincent, C., Rousset, F., Klein, B., Bataille, R., Flacher, M., Durie, B.G., and Revillard, J.P. 1988. Estrogen and progesterone receptors in some human myeloma cell lines and hybridomas. *J. Steroid Biochem.* 30:363-367.
- Danel, L., Souweine, G., Monier, J.C., and Saez, S. 1983. Specific estrogen binding sites in human lymphoid cells and thymic cells. *J. Steroid Biochem.* 18:559-563.
- Dayan, M., Zinger, H., Kalush, F., Mor, G., Amir-Zaltzan, Y., Kohen, F., Stoeber, Z., and Mozes, E. 1997. The beneficial effects of treatment with tamoxifen and anti-estradiol antibody on experimental systemic lupus erythematosus are associated with cytokine modulations. *Immunol.* 90:101-108
- De Carli, M., D'Elia, M., Mariotti, S., Marocci, C., Pinchera, A., Ricci, M. 1993. Cytolytic T cells with TH1-like cytokine profile predominate in retrobulbar lymphocytic infiltrates of Grave's ophthalmopathy. *J. Clin. Endocrinol. Metab.* 77: 1120-1124.
- Dotta, F., and Eisenbarth, G. S. 1989. Type 1 diabetes mellitus: a predictable autoimmune disease with interindividual variation in the rate of β cell destruction. *J. Immunol. Immunopathol.* 50 (1): S85-S95.
- Drappa, J., Brot, N., and Elkon, K.B. 1993. The Fas protein is expressed at high level on CD4⁺ CD8⁺ thymocytes and mature lymphocytes in normal mice but not in the lupus-prone strain MRL lpr/lpr. *Proc. Natl. Acad. USA.* 90:10340-10344.
- Dudley, D. J., Chen, C. J., Mitchell, M. D., Daynes, R. A., and Araneo, B. A. 1993. Adaptive immune response during murine pregnancy: pregnancy-induced regulation of lymphokine production by activated T lymphocytes. *Am. J. Obstet. Gynecol.* 168:1155-1163.
- Duke, R.C., and Cohen, J.J. 1986. IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Rev.* 5: 289-
- Duke, R.C., Chervenak, R., and Cohen, J.J. 1983. Endogenous endonuclease-induced DNA fragmentation: An early event in cell-mediated cytolysis. *Proc. Natl. Acad. Sci. USA.* 80:6361-6365

- Duquette, P., and Girard, M. 1993. Hormonal factors in susceptibility to multiple sclerosis. *Current opinion in neurology and neurosurgery*. 6:195-201.
- Duquette, P., Pleiner, J., Girard, M., Charest, L., Senecal-Quevillon, M., and Masse, C. 1992. The increased susceptibility of women to multiple sclerosis. *Can. J. Neurol. Sci.* 19 (4):466-471.
- Edinger, D., and Garrett, T.J. 1972. Studies of the regulatory effects of the sex hormone on antibody formation and stem cell differentiation. *J. Exp. Med.* 136: 1098-1116
- Emlen, O.W., Niebur, J., and Kadera, R. 1994. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J. Immunol.* 152: 3685-3692.
- Epe, B., Harttig, H., Schiffmann, D. and Metzler, M. 1989. Covalent binding to microtubular proteins as a possible cause of the aneuploidy and micronucleus formation induced by carcinogenic estrogens and other carcinogens. *Environ. Mol. Mutagen.* 14:57.
- Fassler, R., Dietrich, H., Kromer, G. 1988. The role of testosterone in spontaneous autoimmune thyroiditis of obese strain chickens. *J. Autoimmunity* 1: 97-108.
- Fox, H. S., Bond, B. L., and Paslow, T. G. 1991. Estrogen regulation the γ -IFN promoter. *J. Immunol.* 146:4362-4367.
- Gajewski, T. F.; Schell, S.R., Nau, G. and Fitch, F. W. 1989. Regulation of T-cell activation: Differences among T-cell subsets. *Immunol review* 111: p79-110.
- Gilmore, W., Weiner, L. P., and Correale, J. 1997. Effect of estradiol on cytokine secretion by proteolipid protein-specific T cell clones isolated from multiple sclerosis patients and normal control subjects. *J. Immunol.* 158:446-451.
- Giordano, C., De Maria, R., Todaro, M., Richiusa, P., Papoff, G., Ruberti, G., Bagnasco, M., Testi, R., and Galluzzo, A. 1997. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. *Science*. 275 (14): 960-963.
- Graninger, M.B. 1993. Transcriptional overexpression of the proto-oncogene bcl-2 in patients with systemic lupus erythematosus. *Wien Klin Wochenschr.* 104: 205-207.
- Griensven, M. Van, Bergijk, E.C., Baelde, J.J., Heer, D.E. and Bruijn, J.A. 1997. Differential effects of sex hormones on autoantibody production and proteinuria in chronic graft-versus-host diseases-induced experimental lupus nephritis. *Cli. Exp. Immunol.* 107:254-260.
- Grossman, C. J. 1984. Regulation of the immune system by sex steroids. *Endocrine review.* 5: 435-455.
- Grossman, C. j., Sholiton, L. J., and Nathan, P. 1979. Rat thymic estrogen receptor. I. Preparation, location and physiochemical properties. *J. Steroid Biochem.* 11:1233-1240.
- Hadley, M. 1996. *Endocrinology*. (Fourth edition). Prentice-Hall, Inc. Simon & Schuster / A viacom Company. NJ.
- Haruki, Y, Seiki, K., Enomoto, T., Fujii, H. and Sakabe, K. 1983. Estrogen receptor in the "non-lymphocytes" in the thymus of the ovariectomized rat. *Tokai J. Exp. Clin. Med.* 8:31-39.
- Hassuneh, M.R. 1996. Role of autocrine growth factors in the tumorigenic transformation of T cells. *VA tech.* p 12

- Hawkins, T. A., Gala, R. R., and Dunbar, J. C. 1996. The lymphocyte and macrophage profile in the pancreas and spleen of NOD mice: percentage of interleukin -2 and prolactin receptors on immunocompetent cell subsets. *J. Reprod. Immunol.* 32:55-71.
- Hayashi, Y., Haneji, N., and Hamano, H. 1996. Cytokine gene expression and autoantibody production in sjogren's syndrome of MRL/lpr mice. *Autoimmunol.* 23: 269-277.
- Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F. 1949. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis: preliminary report. *Proc. Staff Meetings Mayo Clin.* 24:181-97.
- Hench, P. S. 1938. The ameliorating effect of pregnancy on chronic atrophic (infectious rheumatoid) arthritis, fibrositis, and intermittent hydrarthrosis. *Pro. Staff. Meetings Mayo Clin.* 13:161-7.
- Ho, P. C., Tang, G.W.K., and Lawton, J.W.M. 1991. Lymphocyte subsets in patients with estrogen deficiency. *J. Report Immunol.* 20 (1): 85-91
- Hockenbery, D., Zutter, M., Hickey, W., Nahm, M., and Korsmeyer, S.J. 1990. Bcl-2 protein is topographically restricted in tissues characterized by apoptotic death. *Proc. Natl. Acad. Sci. USA* 88, 6961-6965.
- Homo-Delarche, F., Fitzpatrick, F., Cristeff, N., Nunez, E.A., Bach, J.F., and Dardenne, M. 1991. Sex steroids, glucocorticoids, stress and autoimmunity. *J. Ster. Biochem. Mol. Biol.* 1991. 40: 619-637.
- Huang, Y.P., Perrin, L.H., Miescher, P.A., and Zubler, R.H. 1988. *J. Immunol.* 141: 827-833
- Huang, Z., Gao, J., Wickham, L.A., and Sullivan, D.A. 1995. Influence of gender and androgen treatment on TGF- β 1 mRNA levels in the rat lacrimal gland. *Invest. Ophthalmol. Vis Sci.* 35:S991.
- Huygen, K., and Palfliet, K. 1984. Strain variation in interferon- γ production of BCG-sensitized mice challenged with PPD. II. importance of one major autosomal locus and additional sexual influences. *J. Immunol.* 85:75-81.
- Inman, R. D. 1978. Immunologic sex differences and the female predominance in systemic lupus erythematosus. *Arthritis Rheum.* 21:849-852.
- Ivashkiv, L.B. 1996. Cytokine expression and cell activation in inflammatory arthritis. *Adv. Immunol.* 63: 337-376.
- Janeway, C. A. Jr., Carding, S., Jones, B., Murry, J., Portoles, P., Rasmussen, R., Rojo, J., Kaizawa, K., West, J., and Bottmly, K. 1988. CD4+ T cells: Specificity and function. *Immunol. Rev.* 101: 39-80
- Janeway, C.A. Jr, and Kim, B. 1994. Signals and signs for lymphocyte responses. *Cell.* 76: 275-285.
- Jungers, P., Nahoul, K., Pelissier, C., Dougados, M., Tron, F., and Bach, J.F. 1982. Low plasma androgens in women with active or quiescent systemic lupus erythematosus. *Arthritis Rheum.* 25:454-457.
- Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. *Annu. Rev. Immunol.* 14:207-32.

Kahaleh, M.B., and LeRoy, E.C. 1989. *Ann. Intern. Med.* 110: 446-450

Kakkanaiah, V.N., Seth, A., Nagarkatii, M., and Nagarkatii, P.S.1990. Autoreactive T cell clones isolated from normal and autoimmune susceptible mice exhibit both Th1 and Th2 cells. *Clin.Immunol. Immunopath.* 57:148-162.

Kawashima, I., Sakabe, K., Seiki, K., Fujii-Hanamoto, H., Akatsuka, A., and Tsukamoto, H. 1991. Localization of estrogen receptor cells, with special reference to thymulin (FTS)-producing cells in female rat thymus. *Thymus* 18:79-93.

Kawashima, I., Seiki, K., Sakabe, K., Ihara, S., Akatsuka, A., and Katsumata, Y. 1992. Localization of estrogen receptor and estrogen receptor-mRNA in female mouse thymus. *Thymus.* 20:115-121.

Kearney, E. R., Theresa, L. W., Robert, W. K., Philip, A. M., Dennis, Y. L., Jeffrey, A. B., and Marc, K. J. 1995. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD18 costimulation and inhibited by CTLA-4. *J. Immun.* 155: 1032-1036.

Khangure, M. S., Dingle, P. R., Stephenson, J., Bird, T., Hall, R., and Evered, D. C. 1977. A long term follow up of patients with autoimmune thyroid disease. *Clin. Endocrinol.* 6:41-48.

Korsmeyer, S. J. 1992. Bcl-2: a repressor of lymphocyte death. *Immunol. Today* 13 (8)285-287.

Krammer, P.H. 1993. Lymphocyte activation induced apoptosis by the APO-1 cell surface receptor. *Apoptosis.* Plenum Press. New York, 237-240.

Kuby, J. 1994. *Immunology.* Second edition. W.H.Freeman and Company. New York.

Kuchroo, V.J., Das, M.B., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., and Glimcher, L.H. . 1995. B7-1 and B7-2 cosimulatory molecules activate differentially the TH1/TH2 developmental pathways: Application to autoimmune disease therapy. *Cell.* 80: 707-718.

Kumagai, S., Yanagida, H., and Tsujimoto, Y. 1993. Increased expression of bcl-2 protein in peripheral B cells of patients with systemic lupus erythematosus (SLE). *Arth. Rheum.* 36: S76.

Kyprianou, N., and Issacs, J.T. Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. *Mol. Endocr.* 1989. 7:480-484.

Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varle, P., Joulin, V., and Kahn, A.1996. Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nature Medicine* 2:80-86.

Lahita, R.G. 1993. The effect of sex hormones on the immune system in pregnancy. *Am. J. Reprod. Immunol.* 28 (3/4): 136-137.

Lahita, R. G., Bradlow, H. L., Ginzler, E., Pang, S., and New, M. 1987. Low plasma androgens in women with systemic lupus erythematosus. *Arthritis Rheum.* 30:241-248.

Lahita, R. G., Bradlow, H. L., Kunkel, H. G., and Fishman, J. 1979. Alterations of estrogen metabolism in systemic lupus erythematosus. *Arthritis Rheum.* 22: 1195.

Lahita, R. G., Bradlow, L., Fishman, J. 1981. Increased 16 β -hydroxylation of estradiol in systemic lupus erythematosus. *J. Clin.Endocrinol.* 53:174-178.

- Lemm, G., and Warnatz, H. 1986. *Clin. Exp. Immunol.* 64, 71-79.
- Magnusson, U. 1991. In vitro effects of prepartum concentrations of oestradiol-17 β on cell-mediated immunity and phagocytosis by porcine leukocytes. *Vet. Immunol. Immunopathol.* 28: 117-126.
- Masi, A. 1995. Sex hormones and rheumatoid arthritis cause or effect relationships in complex pathophysiology? *Clin. Exp. Rheumatol.* Mar.-Apr; 13 (2): 227-40.
- Masi, A. T., Josipovic, D. B., and Jefferson, W. E. 1984. Low adrenal androgenic-anabolic steroids in women with rheumatoid arthritis (RA): gas-liquid chromatographic studies of RA patients and matched normal control women indicating decreased 11-deoxy-17-ketosteroid excretion. *Semin. Arthritis Rheum.* 14:1-23.
- Masi, A. T., Feigenbaum, S. L., Chatterton, R. T., and Cutolo, M. 1995. Integrated hormonal-immunological-vascular (HIV triad) systems interactions in rheumatic diseases. *Clin. Exp. Rheumatol.* 13:206-16.
- Mathur, S., Mathur, R.S., Goust, J.M., Williamson, M.O., and Eidenberg, M.M. 1979. Cyclic variations cell subpopulations in the human menstrual cycle: Correlations with progesterone and estradiol. *Clin. Immunopathol.* 13:246-253.
- Matter, A. 1979. Microcinematographic and electron microscopic analysis of target cell lysis induced by cytotoxic T lymphocytes. *Immunol.* 36:179-190.
- McIntyre, T.M., Klinman, D.M. Rothman, P., Lugo, M., Dasch, J.R., Mond, J.J., and Scapper, C.M. 1993. Transforming growth factor β 1 selectively stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *J. Exp. Med.* 177: 1031-1037.
- Miossec, P., Naviliat, M., D'Angeac, A. D., Sany, J., and Banchereau, J. 1990. Low levels of interleukin-4 and high levels of transforming growth factor β in rheumatoid synovitis. *Arth. Rheum.* 33. 1180-1187.
- Morahan, G., Allison, J., and Miller, J.F.A.P. 1989. Tolerance of class I histocompatibility antigens expressed extrathymically. *Nature.* (London) 339: 22-625.
- Mori, K., Kobayashi, S., Inobe, M., Jia W.Y. Tamakoshi, M., Miyazaki, T., and Uede, T. 1994. In vivo cytokine gene expression in various T cell subsets of the autoimmune MRL/Mp-lpr/lpr mouse. *Autoimmunity* 17, 49-57.
- Morgan, D. D., and Grossman, C. J. 1984. Analysis and properties of the cytosolic estrogen receptor from rat thymus. *Endocr. Res.* 10:193-207.
- Mosmann, T. R., and Coffman, R.L. 1989. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different function properties. *Ann. Rev. Immunol.* 7: 145-173.
- Mosmann, T. R., and Sad, S. 1996. The expanding universe of T cell subsets: TH1, TH2 and more. *Immunol. Today* 17:138.
- Mustafa, M., Diener, P, Hojeberg, B., Van der Meide, P., and Olsson, T. 1991. T cell immunity and interferon- γ secretion during experimental allergic encephalomyelitis in Lewis rats. *J. Neuroimmunol.* 31: 165-177.
- Mysler, E., Bini, P., Drappa, J., Ramos, P., Friedman, S., Krammer, P.H., and Elkon, K.B. 1994. The apoptosis/Fas protein in human systemic lupus erythematosus. *J. Clin. Invest.* 93: 1029-1034.

- Nilsson, B., Bergqvist, A., Lindblom, D., Ljungberg, O., Sodergard, R., and von Schoultz, B. 1986. Characterization and localization of specific oestrogen binding in the human thymus. *Gynecol. Obstet. Invest.* 21:150-157.
- Novotny, E. A., Raveche, E. S., Sharrow, S., Ottinger, M., and Steinberg, A. D. 1983. Analysis of thymocyte subpopulations following treatment with sex hormones. *Clin. Immunol. Immunopath.* 28:205-217.
- Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature.* 346: 806-809.
- Ogawa, N., Dang, H., and Talal, N. 1995. Apoptosis and autoimmunity. *J. Autoimmunity.* 8: 1-19
- Okayasu, I., Kong, Y. M., and Rose, N. R. 1981. Effect of castration and sex hormones on experimental autoimmune thyroiditis. *Clin. Immunol. Immunopathol.* 20:240-245.
- Olsen, N. J., and Kovacs, W. J. 1996. Gonadal steroids and Immunity. *Endocrinol. Review.* 17:(4) 369-384.
- Olsen, N. J., Watson, M. B., Henderson, G. S., and Kovacs, W. J. 1991. Androgen deprivation induces phenotypic and functional changes in the thymus of adult male mice. *Endocrinology* 129:2471-2476.
- Paul, W. 1991. Interleukin-4: A prototypic immunoregulatory lymphokine. *Blood* 77: 1859-1870.
- Poser, S., and Poser, W. 1983. Multiple sclerosis and gestation. *Neurology.* 33:1422-1427.
- Rockett, M.K., Bonomo, A., Scott, D.E., Cannella, B., Raine, C.S., Shevach, E.M., and Rocken, M. 1994. Cytokine induced immune deviation as a therapy for inflammatory autoimmune diseases. *J. Exp. Med.* 180: 1961-1966.
- Reuber, M. D., and Golver, E. L. 1976. Role of age and sex in chronic thyroiditis in rats fed 3-methyl-4-dimethyl-aminoazo-benzene. *Vet. Pathol.* 13:295-302.
- Reynolds, D.S.; Boom, W. H., and Abbas, A. K. 1987. Inhibition of B lymphocyte activation of interferon- γ . *J. Immunol.* 139: 767-773
- Rose, L.M., David, S.L., and David, A.I. 1994. Bcl-2 and Fas molecules which influence apoptosis. A possible role in systemic lupus erythematosus? *Autoimmunol.* 17: 271-278
- Roubinian, J. R., Talal, N., Greenpan, J. S., Goodman, J. R., and Siiteri, J. K. 1978. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J. Exp. Med.* 147:1568-1583.
- Roubinian, J. R., Papoian, R., and Talal, N. 1977. Androgenic hormones modulate auto-antibody responses and improve survival in murine lupus. *J. Clin. Invest.* 59 (6):1066-1070.
- Russell, J.H. 1981. Internal disintegration model of cytotoxic lymphocyte-mediated cytotoxicity. *Biol. Rev.* 56:153-
- Russell, J.H. 1983. Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Res.* 72:97-
- Russell, J.H., Masakowski, V.R., Rucinsky, T., Phillips, G. 1982. Mechanisms of immune lysis 3. Characterization of the nature and kinetics of the cytotoxic T lymphocyte-induced nuclear lesion in the target. *J. Immunol.* 128:2087-2094.
- Russell, J.H., and Dobos, C.B. 1980. Mechanisms of immunolysis 2. CTL-induced disintegration of the target begins within minutes of cell contact. *J. Immunol.* 125:1256-1261.

- Sarvetnick, N, and Fox, H.S. 1990. Interferon-gamma and the sexual dimorphism of autoimmunity. *Mol. Biol. Med.* 7: 323-331.
- Schapira, K., Poskanzer, D. C., Newell, D. J., and Miller, H. 1966. Marriage, pregnancy and multiple sclerosis. *Brain.* 89:419-428.
- Screpanti, I., Morrone, S., and Meco, D. 1989. Steroid sensitivity of thymocytic subpopulations during intrathymic differentiation. Effect of 17 β -estrodinol and dexmathasone on subsets expressing T cell antigen receptor or IL-2 receptor. *J. Immunol.*142:3378-3383.
- Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S.J. 1991. bcl-2 inhibits multiple forms of apoptosis but not negtive selection in thymocytes. *Cell* 67:879-888.
- Shira, A., Conover, J., and Klinman, D. M. 1995.Increased activation and altered ratio of interferon-gamma : interleukin-4 secreting cells in MRL-lpr/lpr mice. *Autoimmunity.* 21, 107-116.
- Silverstone, A. E., Frazier, D. E.,Jr. and Gasiewicz, T. A. . 1994. Alternate immune system targets for TCDD:lymphocyte stem cells and extrathymic T-cell development. *Exp. Clin. Immunol.* 11:94-101.
- Smithson, G., Medina, K., Ponting, I., and Kincade, P.W. 1995. Estrogen suppresses strmal cell-dependent lymphopoisis in culture. *J.Immunol.* 155: 3409-3417.
- Spector, T. D. 1989. Sex hormone measurements in rheumatoid arthritis. *Br. J. Rheumatol.* 28 (Suppl 1): 62-68, Discussion 69-71.
- Strasser, A., Whittingham, S., and Vaux, D.L. 1991. Enforced bcl-2 expression in B lymphoid cells prolongs autoimmune diseases and elicits autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 88:861-8665
- Stimson, W.H. 1988. Oestrogen and human T lymphocytes: present of specific receptors in the T-suppressor/cytotoxic subest. *Scandinavian J. Immunol.* 28:345-350.
- Sullivan, D.A., and Edwards, J.A. 1997. Androgen stimulation of lacrimal gland function in mouse models of Sjogren's syndrome. *J.Ster. Biochem Mol. Biol.* in press.
- Sweeney, W. J. 1955. Pregnancy and multiple sclerosis. *Am. J. Obstet. Gynecol.* 66:124-130.
- Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. 1994. Generalized lymphoproliferative diseases in mice, cause by a point mutation in the Fas ligand. *Cell* 76 (March):969-976.
- Talal, N. 1979. Systemic lupus erythematosus, autoimmunity, sex and inheritance. *N. Engl. J. Med.* 301(15):838-839.
- Talal, N. 1981. Sex steroid hormones and systemic lupus erythematosus. *Arthritis Rheum.* 24 (8):1054-1056.
- Talal, N., and Ansar Ahmed, S. 1987. Immunomodulation by hormones--An area of growing importance. *J. Rheumatol.* 14 (2): 191-193
- Trauth, B.C., Klas, C., and Peters, A.M.J. 1989. Monoclonal antibody mediated tumor regression by induction of apoptosis. *Science.* 254:301-304.
- Tucker, R.W., and Barrett, J.C. 1986. Decreased number of spindle and cytoplasmic microtubules in hamster embryo cells treated with a carcinogen, diethystibestrol. *Cancer Res.* 46: 2088

- Ullrich, A., and Schlessinger, J. 1990. Signal transduction by receptor tyrosine kinase activity. *Cell*. 61: 203-212.
- Vaux, D.L., Cory, S., and Adams, J.M. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*. 335: 440-442.
- Verthelyi, D., and Ansar Ahmed, S. . 1994. 17 β -estradiol but not 5 α -dihydrotestosterone augment antibodies to double stranded deoxyribonucleic acid in non-autoimmune C57BL/6J mice. *Endocrinology*. 135: 2615-2622.
- Viselli, S. M., Stanziale, S., Shults, K., Kovacs, W. J., and Olsen, N. J. 1995. Castration alters peripheral immune function in normal male mice. *Immunology*. 84:337-342.
- Walker, S. E.; Besch-Williford, C. L., and Keisler, D. H. 1994. Accelerate deaths from systemic lupus erythematosus in NZB \times NZW F1 mice treated with the testosterone-blocking drug flutamine. *J. Lab. Clin. Med.* 124: 401-407.
- Watanabe, D., Suda, T., Hashimoto, H., and Nagata, S. 1995. Constitutive activation of the Fas ligand gene in mouse lymphoproliferative disorders. *EMBO J.* 14(1):12-18.
- Watanabe-Fukunaga, R., Brannan, C.I., Itoh, N., Yonehara, S., Copeland, N.G., Jenkins, N.A., and Nagata, S. 1992. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J. Immunol.* 148:1274-1279.
- Webb, S., Morris, C., and Sprent, J. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell*. 63: 1249-1256.
- Whaley, K., and Buchanan, W. W. 1980. Sjogrens' syndrome and associated diseases, *Clinical immunology*. Edited by CW Parker. Philadelphia, W. B. Saunders. p 632.
- Wheeler, W.J., Cherry, L.M., Downs, T., and Hsu, T.C. . 1986. Mitotic inhibition and euploidy induction by naturally occurring and synthetic estrogens in Chinese hamster cell in vitro. *Mutation Res.*
- Wilder, R.L. 1995. Neuroendocrine-immune system interactions and autoimmunity. *Annu. Rev. Immunol.* 13:307-338
- William, C.G., and James, A. M. 1986. Effect of testosterone treatments for varying periods on autoimmune development and on specific infiltrating leukocyte populations in the thyroid gland of obese strain chickens. *Clin. Immunol. Immunopathol.* 39:464-478.
- Williams, E. D., and Doniach, I. 1962. Postmortem incidence of thyroiditis. *J. Pathol. Bacteriol.* 83: 255-264.
- Wingrave, S. J. 1978. Reduction in incidence of rheumatoid arthritis associated with oral contraceptives. *Lancet* 1(8068):829
- Wyllie, A.H. 1993. Apoptosis. *Br J. Cancer* 67:205-208.
- Wogesen, L., Lee, M.S., and Sarvetnick, N. . 1994. Production of interleukin-10 by islet cells accelerates immune-mediated destruction of β cells in Non-obese diabetic mice. *J. Exp. Med.* 179: 1379-1384.
- Yamamoto, K.R. 1985. Steroid receptors regulated transcription of specific genes and gene networks. *Annual Review of Genetics* 19: 209-252.

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