EFFECTS OF ESTROGEN ON THE B CELL FUNCTIONS OF NORMAL MICE

by

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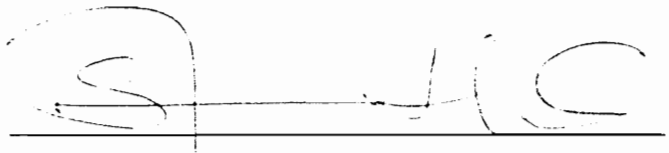

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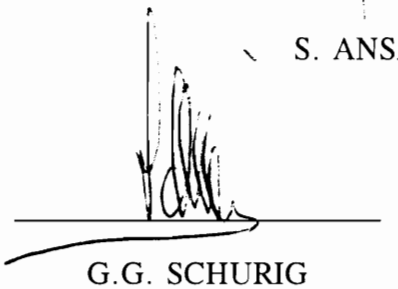

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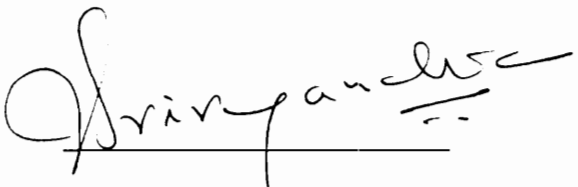

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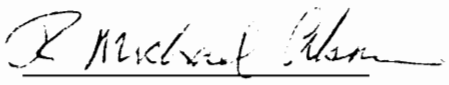

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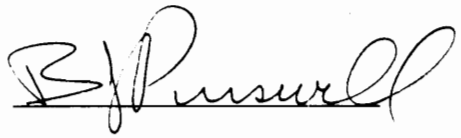

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Daniela I. Verthelyi

Committee Chairman: S. Ansar Ahmed
Veterinary Medical Science

(Abstract)

It is now recognized that reproductive hormones such as estrogen influence not only classical targets (eg. reproductive tissues), but may also act on non-classical target sites such as the immune system. A better understanding of the effects of estrogen on the immune system is of paramount importance since: (i) increasing numbers of women around the world take estrogen-containing oral contraceptives, some times for most of their reproductive life; (ii) estrogen is often prescribed as a replacement therapy to postmenopausal women; and (iii) a large number of pesticides, insecticides, and phytoestrogens (plant-derived estrogens) have been found to have hormone disrupting effects as evidenced by altered development of reproductive and immune functions in wild species.

The precise effects of estrogen on the normal immune system are not well known. The overall objective of this work has been to better understand the role of sex hormones on the B cell function of normal mice. It is hoped that this will lead to an improved understanding of the pathogenesis and treatment of immune-related disorders such as autoimmune diseases and cancer. These studies were accomplished by parenteral administration of estrogen to nonautoimmune C57BL/6 mice, a widely used strain in immunology. In these mice, we found that treatment with estrogen, but not 5α-dihydrotestosterone, induced the expression of a wide variety of IgG and IgM autoantibodies and heteroantibodies that are associated with autoimmune and infectious diseases. These include antibodies to cardiolipin and other membrane phospholipids, dsDNA and acetone-killed *Brucella abortus* strain RB51. Importantly, the expression of anti-dsDNA and anti-

cardiolipin antibodies was sustained for several months after the removal of the exogenous source of estrogen. This indicates that the immunomodulatory effect of estrogen is long-lasting. These antibodies have a marginal degree of crossreactivity with other antigens and belong mainly to IgG2b subisotype.

These findings were confirmed at the cellular level, where we have shown that estrogen-treated mice have increased numbers of plasma cells in the spleen, and that these plasma cells actively secrete IgM and IgG immunoglobulins as assessed by ELISPOT. Further, higher immunoglobulin yield per cell was evident in estrogen-treated than in placebo-treated controls in the spleen and bone marrow. Interestingly, we found that splenic lymphocytes had an increase in antibody-forming cells for all specificities tested. Active antibody-forming cells from bone marrow preferentially recognized autoantigens, cardiolipin and dsDNA.

Functional analysis on the viability of the splenic lymphocytes showed that *in vivo* exposure to estrogen resulted in: (a) increase in the proportion of cells dying by apoptosis, and (b) an increased proportion of lymphocytes that were actively proliferating as assessed by cell cycle analysis. Culturing of B cells in the absence of any deliberate stimulus showed the B cells from estrogen-treated mice underwent active proliferation and resisted death by apoptosis more compared to controls. We also found that despite the autoproliferative character of splenic B cells, they were able to respond adequately to stimulation with anti-CD40 antibodies, IL-4 and lipopolysaccharides. B cells from mice treated with estrogen had a marked reduction in their susceptibility to apoptosis when cultured in the presence of such stimuli.

Together these studies indicate that normal mice exposed to estrogen may express a variety of autoantibodies, show signs of B cell hyperactivity, have defects in susceptibility of B cells to apoptosis as well as the ability to proliferate in the absence of stimulation. It is hoped that these studies would enhance our understanding of the immunomodulatory role of estrogen in health and in a wide range of disorders such as autoimmune and cancer disorders.

This dissertation is dedicated to

my husband, ***Eduardo Romano*** *whom I love and respect, for all his support and for making me laugh even when experiments didn't work;*

my daughter, ***Ari****, for all the joy and for reminding me daily that learning is a lot of fun;*

my parents, ***Renata and Johnny,*** *who instilled in me the love for learning;*

my big brother, ***Roberto,*** *who at last admitted that I might not be "that lazy" after all.*

I am very lucky to have you all.

Also, to Vera, Chuck and Dale.

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There is a long list of people I would like to thank for helping me in this step of the road to become a scientist. First and foremost, my thanks to my advisor, Dr Ansar Ahmed, for his relentless support and for countless hours of passionate thinking, imagining and debating. I know someday I will also thank him for endless drafts and for always pushing for a little bit more. I would like to thank also the committee members, Dr Gerhardt Schurig and Dr Nammalwar Sriranganathan, for their support, guidance, and constructive criticism. Dr Beverly Purswell and Dr Michael Akers for their interest and patience. Special thanks to Dr Klaus Elgert for teaching me the rudimentaries of immunology and for his assistance and encouragement. I am grateful for the help received from Dr Bill Chikering, Dr Tom Caceci, Dr John Robertson, and Dr Eric Wong. My gratitude to Dr Gordon Carter for patiently correcting the draft of this thesis. My appreciation to Dr Robert Lahita for agreeing to serve as my external examiner.

The road was significantly facilitated by all the people at the Veterinary Medicine Research Center (AKA, Center for Molecular Medicine and Infectious Diseases). I would specially like to thank Jane Walsh, for her very competent assistance. Also, my good friends, Dr Chris Ward and Dr Lynn McGonagle, for their attitude and confidence; Dr Oscar Pintado, without whom I might have never started; Dr Silvio Cravero for hours of "mindboggling" discussions. Also, to Dr Mat Walker and Dr Dave Aleva for setting the standards. My thanks go to my friends and coworkers at

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LIST OF ABBREVIATIONS:

7-AAD	7-amino-actinomycin D
ABS	Adult bovine serum
ACTH	Adrenocorticotropin
APS	Antiphospholipid syndrome
BCR	B cell receptor
Br-ME	Bromelin-treated mouse erythrocytes
BSA	Bovine serum albumin
CD	Cluster of differentiation
CMI	Cell mediated immunity
CNS	Central nervous system
Con A	Concanavalin A
CRH	Corticotropin releasing factor
DHEA	Dehydroepiandrosterone
DHT	Dehydrotestosterone
DNA	Deoxyribonucleic acid
ds	double stranded
DTH	Delayed type hypersensitivity
E2-O	Estrogen-orchiectomized
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme linked immunoassay
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GH	Growth hormone
HSA	Heat stable antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LH	Luteinizing hormone
LPS	Lipopolysaccharide
mIg	membrane immunoglobulin or surface immunoglobulin
MRL/lpr	MRL/Mp-lpr/lpr
OC	oral contraceptive
OD	optical density
OVA	Ovalbumin
NK	Natural killer
P	placebo
P-O	Placebo-orchiectomized
P-S	Placebo-sham orchiectomized
PFC	Plaque forming cells
Pg	Prostaglandin
PHA	Phytohemagglutinin

PI	Propidium iodide
PRL	prolactin
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RBC	red blood cells
RPMI	Roswell Park Memorial Institute
sIg	membrane immunoglobulin or surface immunoglobulin
SLE	Systemic lupus erythematosus
SRBC	sheep red blood cells
ss	Single stranded
SS	Sjögren syndrome
TGF-β	Transforming growth factor-β

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CHAPTER 1:

INTRODUCTION

For many years attempts to understand how the body worked were addressed by studying each body system independently. Although this reductionist approach allowed us to understand some of the basic phenomena, it disregarded the intricate interaction among the different biologic systems in the body. The immune system, for example, was thought to operate in an autonomous fashion. Over the past few years, our understanding of the immune system has improved, and it is now clear that the immune system interacts with other body systems such as the endocrine system. Sex hormones have been known for many years to influence the immune system. References on the effects of sex hormones on the immune system date as far back as 1898, when Cazolari, an Italian anatomist, noted that gonadectomy reduced the size of the thymus. However, since at the time the thymus was considered a superfluous organ, the observation was overlooked and research in this area, except for a few isolated clinical references, was neglected. The preponderance of autoimmune diseases in women and the gender-related differences in immunocapabilities have reignited interest in this area. The complex bidirectional interactions of sex hormones and the immune system are now becoming well recognized by scientists and clinicians. The studies in this thesis are focused on the effects of a female sex hormone, estrogen, on B lymphocytes.

A better understanding of the effects of estrogen on the immune system is of paramount importance since: (i) Increasing numbers of women around the world take estrogen-containing oral contraceptives, some times for most of their reproductive life; (ii)

estrogen is often prescribed as a replacement therapy to postmenopausal women; and (iii) a large number of pesticides and insecticides have been found to have hormone disrupting effects which result in altered development as well as reproductive and/or immune function of a range of wild species.

Previous studies have shown that sex hormones influence the course of systemic autoimmune diseases in both humans and animals. However, information on how sex hormones in general, and estrogen in particular, might affect the homeostasis of the immune system of normal individuals is limited. The purpose of this work has been to improve our understanding of the role of sex hormones in the normal immune system in the hope that this will lead to improved understanding of the pathogenesis and treatment of immune-related disorders such as autoimmune diseases and cancer. This was accomplished by exposing nonautoimmune C57BL/6 mice to exogenous hormones through subcutaneous time-release implants. C57BL/6J mice are currently considered as non-autoimmune, and have been employed as a control in numerous immunologic studies. However, with age, C57BL/6J mice have been shown to develop inflammatory lesions in salivary glands, kidney, pancreas, lungs and liver. These autoimmune-like lesions have been reported to appear in as early as 6 month of age. Interestingly, the expression of these lesions does not appear to be influenced by gender, except for those in the salivary glands which were more prevalent in females. (Hayashi et al.,1989; Ansar Ahmed , 1994). Since autoimmune diseases are multifactorial (genetic, environmental, hormonal, infectious factors, etc.), it was felt that C57BL/6J mice would represent a good model to study the influence of sex steroids on healthy individuals who may have some predisposing factors for autoimmune disease in their background.

The results obtained are described in: Chapters 3, 4, and 5 which address the estrogen-induced expression of autoantibodies, including the characterization of the antibodies with regard to kinetics of expression, isotype and subisotype distribution, and cofactor binding requirements. Chapter 6, deals with the effects of estrogen on the number of antibody-secreting cells from different organs utilizing an ELISPOT system. Chapter 7, describes the ability of B cells from estrogen-treated mice to respond to a range of stimuli. In particular this chapter will focus on examining the B cells relative to their ability to proliferate upon stimulation, their susceptibility to apoptosis and entry into cell cycle. Chapter 8, characterizes the B cell response of estrogen-treated mice that are vaccinated with *B. abortus* strain RB51, a facultative intracellular bacteria. Chapter 9, includes overall conclusions and attempts to relate the findings to the current literature.

CHAPTER 2:

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1. The role and function of B cells:

A primary function of the immune system is to defend the body against innumerable antigens that constantly enter the body. This defense mechanism involves the interplay of a wide range of cell types (lymphocytes, macrophages, dendritic cells, natural killer cells, among others). At the center of this complex system are the lymphocytes, which are capable of orchestrating a specific response to a defined antigen. Two broad types of lymphocytes are recognized: B and T cells. B lymphocytes are characterized by their ability to recognize and produce immunoglobulins against an immense variety of antigens, and also for their ability to "present" the antigen to the T lymphocytes to evoke an amplified and specific response.

a. B cell development:

B lymphocytes are derived from hematopoietic stem cells following a complex set of differentiation events that are only partially understood. Although it seems reasonable to believe that B and T cells arise from a common stem precursor, evidence is limited partly due to the paucity of known phenotypic markers specific for earlier stages of development. In mammals, early B cell development takes place in the bone marrow of adults and in the omentum, liver, spleen, and bone marrow of the fetus (Kamps and Cooper, 1984; Nuñez et al., 1996). In birds, the bursa of fabricius is the site for B cell maturation (reviewed in Gallagher and Osmond, 1991; Marcos et al.,1994; Paul, 1993). The key events in B cell development center on the sequential rearrangement and assembly of the genetic elements that encode the B cell receptor (BCR) (reviewed in Tarlington, 1994). The BCR is an immunoglobulin that consists of 2 heavy and 2 light

chains. These chains have a constant and a variable region. The variable regions on the heavy and light chain recognize the antigen and give the B cell it's specificity. The genetic components (V, D and J elements) that encode these variable portions are not contiguous in the germ line. Their assembly, which is mediated by recombination activation genes (*Rag 1 and Rag 2),* occurs in a quasi-random manner and result in the generation of a highly diverse B-cell repertoire (Schatz et al., 1989; Oettinger et al., 1990; reviewed in Paul, 1993).

Early B cell development is usually divided into 2 main stages: pro-B cells and pre-B cells. The pro-B cell stage is marked by the rearrangement of the V, D and J elements that encode the variable region of the heavy chain and concludes when a productive μ chain is expressed in the cytoplasm. The V-J rearrangement for the light chain occurs in pre-B cells stage, after which the cell expresses IgM on its surface and becomes an immature B cell (reviewed in Paul, 1993).

B cell developmental stages have been associated with the expression of cell surface antigens on their surface. Early in pro-B cell stage, during the rearrangement of the DJ elements of the heavy chain, B cells express CD43 (also called leukosialin). After this, during the V-DJ rearrangement, there is an increase in heat stable antigen (HSA) (Hardy et al., 1991). At this stage, B cell development depends heavily on cell contact with stromal cells and local concentration of IL-7 (Hardy et al., 1991; Tsubata and Nishikawa, 1991). Upon transition to Pre-B, the CD43 surface expression declines, and with it the requirement for IL-7 (Hardy et al.,1991; Funk et al., 1993). During the Pre-B cell stage, class II antigens appear on their surfaces, and the expression of CD45R (B220) increases. Selection of the developing B cells (positive and/or negative) is thought to

occur at this stage (phenotypically characterized by IgM $^{very\ lo}$ and HSA $^{very\ Hi}$; reviewed in Klinman, 1994). Upon the expression of sIgM, the B cells are considered immature B cells. When the B cells expresses IgD and CD23 on their surface, they become mature B cells.

b. B cell activation: lipopolysaccharide, anti-CD40 and anti-IgM antibodies:

After emerging from the bone marrow, the naive B cells enter a quiescent stage until they encounter their specific antigen. Antigens capable of activating B cells can be divided into T cell independent and T cell dependent antigens. Direct activation of B cells by T cell-independent antigens requires crosslinking of the BCR. Since the BCRs on the surface of an individual B cell are identical, the antigen has to express more than one copy of the epitope (reviewed in Noelle and Snow, 1991; Paul, 1993). Most T cell independent antigens are large, multivalent molecules with a long half-life when injected *in vivo* (Mond et al., 1995). Among the antigens capable of eliciting such a response are polysaccharides and lipopolysaccharides which are often found on the surface of bacteria. This mechanism of B cell activation is therefore important in establishing a prompt response to microbial antigens. This type of activation usually does not involve T cell help and may lead to proliferation and formation of plasma cells producing specific IgM antibodies. There is evidence that cytokines are required for full activation of the B cell by T cell independent antigens, but they appear to be furnished by local macrophages and natural killer cells (NK cells) (reviewed in DeFranco, 1993; Mond et al., 1995). No memory B cells are generated upon activation by T cell independent antigens (reviewed in DeFranco, 1993). Interestingly, crosslinking of the BCR is known not only to lead to B cell activation, but also to inactivation or even cell death (please refer to section on

B cell death).

B cell activation by T cell dependent antigens allows B cells to mount responses against antigens, usually proteins, that have only few copies of the relevant epitope and therefore cannot crosslink the BCR. The antigen binds to a specific B cell through their BCR and is subsequently internalized. The B cells then process the antigen and present a portion of it on their surface, in the context of class II major histocompatibility complex (MHC), to the T cells (reviewed in Hodkin and Kehry, 1993; McHeyzer-Williams et al., 1991) (**Figure 2.1**). Subsequently, the stimulated T cells can, in turn, strengthen the B cell response to the antigen by secreting lymphokines (e.g. IL-4 , IL-5, IL-6, or IL-10) and/or by direct contact through CD40-CD40L (or CD30-CD30L; Shanenbeck et al.,1995) interactions (Scott, 1995). Ligation of CD40 has been shown to rescue B cells from undergoing cell death (see below, section 2.d. on B cell death). There are several and often redundant paths for B-T communication, for example, in CD40L-deficient mice, B cells are able to survive, proliferate and differentiate. This indicates that there are alternate pathways to avoid B cell death and elicit differentiation (Renshaw et al., 1994). It has been postulated that subsequent to antigen presentation, T cell-dependent activation of B cells is independent of the presence of antigen-specific BCR. This suggests that the role of the surface immunoglobulin is apparently restricted to the capture and internalization of the antigen (Möller et al.,1991; Hodkin and Kehry., 1993). This would appear to endanger the specificity of the lymphocytic response which could potentially lead to autoimmunity. To avoid non-specific activation of B cells, several mechanisms appear to be in place: (i) BCR crosslinking induces changes in the polymerization of the cell membrane, protein kinase C activation and an influx of calcium, which preactivates the B cell; (ii) activated B cells up-regulate their cell

adhesion molecules, which aids in reducing the gap between them and the helper T cell; and (iii) the up-regulation of CD40 ligand on the T cell surface and the lymphokine secretion are transient. The characteristics of the interaction of the B cells with the T cell determines the fate of the response to the antigen: proliferation, differentiation or even death (reviewed in DeFranco, 1993).

The paths involved in B cell activation through the surface immunoglobulin, are not clear. All B cell surface immunoglobulin (sIg) Ig have a short and conserved cytoplasmic tail. Analogous to the T cell receptor, which is coupled to the CD3 complex, in B cells the surface immunoglobulin is coupled to peptides mb-1 and B29 (Igα and Igβ) which convey the signal to the *src* family of tyrosine kinases. These kinases in turn phosphorylate protein kinase-C and start a cascade of events that lead to increased intracellular calcium, diacylglycerol, and inositol triphosphate, and ultimately to the activation of B cells (reviewed in Scott, 1995).

Naive B cell activation prepares the cell to divide and differentiate into plasma or memory cells. The production of antibodies under these circumstances accounts for the primary antibody response. The presence and combination of the cytokines released and the microenvironment, determines the fate of the B cell (see figure 2.1). Importantly, these cytokines also modulate the isotype switch that occurs during differentiation to plasma cells. IL-4 determines the switch to IgE and IgG1 (mice) or IgG4 (human). IL-5 induces the switch to IgA. GammaIFN has been shown to induce the switch to IgG2a (mice), and TGF-β has been reported to bring on the switch to IgA and IgG2b (reviewed in Paul, 1993; McIntyre et al., 1993). After participating in a primary immune response, most of the activated B cells are quickly removed from the circulation and failure to do

so has been shown to lead to continuous secretion of immunoglobulins and possibly to autoimmunity (Strasser et al., 1991).

c. Memory B cells:

The differentiation of activated B cells into memory B cells occurs in specialized microenvironmental structures, the germinal centers, in spleen and lymph nodes. Upon re-exposure to the same antigen, memory B are capable of rapid proliferation and differentiation into highly specific plasma cells. This has been termed as the anamnestic response. Memory B cells are thought to have very long quiescent lives, have reduced requirements for activation, and to produce very specific, high affinity immunoglobulins of different isotypes. These result from somatic mutation coupled with selection of cells with receptors with heightened avidity for the antigen (reviewed in Linton, 1992). Interestingly, it has been proposed that memory B cells are drawn to recirculate to sites where the likelihood of encountering the antigen is higher (reviewed in Kincade and Gimble, 1993). To date there are no known cell surface markers that are solely expressed in these cells, but memory cells have been shown to have J11d^{lo} and CD44 (a receptor for hyaluronate) on their surfaces (Shevach, 1993). The question on whether persistence of memory B cells depends on the continuous presence of the antigens is currently a subject of debate. This could be achieved by regular contact with low levels of resident microorganism or by long-term presentation of the antigen on dendritic cells. According to this theory, memory B cells would undergo apoptosis in the absence of antigen (reviewed in DeFranco, 1993). This hypothesis is supported by the loss of secondary antibody response after adoptive cell transfer of purified B cells (reviewed in Paul, 1993). The fact that memory B cells have been identified in areas far from the site

where the antigen is located (Bachmann et al., 1994), however, does not support this hypotheses.

d. B cell death:

Cell death occurs at different stages of B cell development and serves to remove defective, redundant or autoreactive lymphocytes, as well as to downregulate the immune response. There are two major paths to cell death: necrosis and apoptosis. Physiologic death is achieved mostly through apoptosis.

Apoptosis is an active form of cell death that can be readily distinguished from necrosis by several morphologic and biochemical features, including cell shrinkage, chromatin condensation and zeiosis (untethering of the cell membrane from the cytoskeleton), with relative conservation of the plasma membrane. Early on in apoptosis, the morphology of the cytoplasmic organelles is conserved, although the functional activity of the mitochondria is soon reduced (reviewed in Williams, 1995). At later stages the membrane blebs out resulting in formation of apoptotic bodies that, *in vivo,* are then phagocytized without-unlike necrosis- provoking inflammation (reviewed in Cohen, 1993; Schwartz and Osborne, 1993).

Although little is still known about the molecular mechanisms that govern apoptosis (see **figure 2.2**) a variety of factors (e.g. lack of required growth factors, crosslinking of the antigen receptor, ligation of fas antigen on the cell surface, exposure to glucocorticoids or radiation) have been found to induce apoptosis of lymphocytes. A number of independent and partially redundant pathways involving several genes and proteins that

effect apoptosis have been identified (e.g. *nur77, p53, c-myc, ICE*). Their function has been shown to be modulated by gene products that facilitate (e.g. *bcl-x_s, bax*), or prevent (e.g. *bcl-2, bclx_L*) apoptosis (Schwartz and Osborne, 1993) **(Figure 2.2)**.

Apoptosis in B cells: Apoptosis is important at several stages in the life of the B cell. Early in B cell development the majority of B cell precursors (approximately 95%) undergo apoptosis (reviewed in Gallagher and Osmond, 1991; Osmond et al., 1994). In the pro- and pre-B cell stage, cells that fail to complete a successful immunoglobulin rearrangement are promptly eliminated (reviewed in Osmond et al., 1994). Once the B cell precursors express their surface BCR, those that are specific for self-antigens undergo clonal elimination (Nemazee and Bürki, 1989; Benhamou et al.,1990; Griffiths et al., 1994; Gottchalk et al., 1994; Norvell et al, 1995).). Apoptosis is not limited to immature lymphocytes, but can be triggered in mature lymphocytes as well (Russell et al., 1991; Hartley et al., 1991; Illera et al., 1993; Chaouchi et al., 1995). In the periphery, after the encounter with the antigen, naive B cells migrate to primary follicles to form a germinal center. At this stage B cells undergo extensive somatic mutation of the variable region of their BCR. B cells that have high affinity BCR are positively selected, while those that have lower affinity BCR undergo apoptosis (reviewed in Krammer et al., 1994). Lastly, apoptosis of activated B cells is thought to be important in the downregulation of the immune response after the antigen has been eliminated (reviewed in Nuñez et al., 1994). B cells stimulated with LPS or anti-μ antibodies have increased expression of Fas on their surfaces (Drappa et al., 1993). The Fas antigen is a 35 kDa transmembrane glycoprotein that has been shown to transduce a direct death signal (Rose et al.,1994) and is one of the signals thought to mediate the downregulation of the immune response. Defects in downregulation of the immune response could lead

to autoimmune disease (Drappa et al., 1993).

Positive and negative selection of B cells appear to be intimately connected to the expression of genes associated with apoptosis. The product of *bcl-2* is an integral membrane protein of the outer mitochondrial membrane, perinuclear membrane and endopasmic reticulum that has been shown to delay to varying degrees apoptotic death induced by growth-factor deprivation, gamma-irradiation, or glucocorticoids (Vaux et al., 1988; Nistani et al., 1993 ;Jackobson, 1994; reviewed in Schwartz and Osbore, 1993; Nuñez et al.,1994). Bcl-2, which is highly expressed in the earlier stages of pro-B cell maturation, is down regulated in pre-B and immature (IgM^+ IgD^-) B cells. At this time, B cells with high affinity for self antigens are eliminated (negative selection). Interestingly, bcl-2 transgenic mice, which express large amounts of Bcl-2 protein, produce various autoantibodies, including anti-dsDNA antibodies, and develop a disease similar to systemic lupus erythematosus (SLE) (Strasser et al., 1991). This suggests that B cell death plays an important role in the prevention of autoimmune diseases. Induction of increased Bcl-2 expression, however, may delay but does not totally prevent negative selection (reviewed in Nuñez et al., 1994). Mature B cells undergoing affinity maturation in the germinal centers have low levels of *bcl-2* expression. The level of Bcl-2, however, increases by crosslinking of the BCR or by ligation of CD40 (Liu et al., 1991; reviewed in Nuñez et al.,1994). Several other genes, sometimes encoding redundant paths, have been associated with blocking apoptosis. In *bcl-2*-deficient mice, antigen-stimulated B cells were able to induce CD40L and interleukin expression in T cells and ultimately prevent their own death (Nakayama et al., 1995). Bcl_{XL}, a member of the Bcl-2 family, has been shown to mediate the CD40-CD40L mediated block of apoptosis in mature B cells (Wang et al.,1995). Bcl_{XL} is also highly expressed in the

bone marrow and has been reported to be important in the survival of early hematopoietic precursors (Motoyama et al., 1995).

Some of the genes that have been linked to apoptosis, like *c-myc* and *p53* have also been associated with proliferation. Interestingly, lymphocytes have been shown to respond differently to stimuli depending on their developmental stage as well as their microenvironment and cooperation with other cells. For example, crosslinking of surface IgM or CD3 induces activation and proliferation of mature B or T cells, respectively (reviewed in Scott, 1995). The same stimuli, however, would induce apoptosis in immature B and T cells (Smith et al., 1989; Griffiths et al., 1994., Gottchalk et al., 1994; Norvell et al, 1995). Also, microenvironmental conditions can determine whether a cell responds to activation by proliferation and differentiation or death. For example, mature B cells stimulated with anti-μ antibodies are driven to apoptosis in the presence of ionomycin, a calcium ionophore (Vasquez et al.,1991). Also, Burkitt lymphoma B cells, which are used as a model for immature B cells, have been reported to undergo apoptosis upon stimulation with suboptimal doses of anti-IgM when their surface CD19 and CD22 are coupled (Chaouchi et al.,1995).

e. B cells in autoimmune disease:

Autoimmune diseases are often distinguished by increased immunoglobulin levels, especially autoantibodies. In systemic autoimmune diseases (e.g. SLE), both humans and murine, the number of B cells that spontaneously secrete immunoglobulins is dramatically increased. The importance of autoantibodies in the development of this disease was illustrated using lpr/lpr B-cell-knockout mice (which lack B cells). These mice have no

signs of vasculitis or kidney damage despite carrying lpr/lpr genes (Shlomchik et al.,1994).

Several possible mechanisms have been proposed to explain the failure of B cell tolerance for "self" antigens. Some of the mechanisms postulated include: First, autoantibodies may be expressed upon exposure to "new self-antigens". These antigen may have been previously "invisible" to the immune system because they were physically sequestered, neoformed, or were previously present in very low concentrations. Exposure to self-antigens could be increased as a result of tissue damage. Second, autoantibodies may be elicited as a response to microbial or other environmental antigens that crossreact with self antigens. This mechanism is usually referred to as molecular mimicry (reviewed in Kotb, 1995; Cohen and Young, 1991). In support of this possibility, the target for autoantibodies are, in many cases ubiquitous, including nucleic acids, ribonucleoproteins, membrane phospholipids or highly conserved heat shock proteins. Third, expansion of autoantibody-forming cells could result from faulty T-B cell interaction (see B cell activation section above). Increased T cell help or decreased T suppressor function have been proposed to lead to autoreactive B cell overactivity. For example, SLE patients have been reported to have increased IL-6 levels (Al-Janadi and Razziudin, 1993). This cytokine has been shown to act on the final stages of B cell differentiation to plasma cells and increase the immunoglobulin secretion, mainly IgM (reviewed in Durum and Oppenheim, 1993). Similarly, the increased production of IL-10 in rheumatoid arthritis, Sjögren syndrome and SLE patients is thought to play a role in B cell hyperactivity and autoimmunity (Llorente et al., 1994) Fourth, B cells may have skewed responses to normal T cell signals (Adachi et al., 1993; reviewed in Eisenberg et al., 1994). Overactive B cells may be polyclonal or respond only to certain autoantigens Klinman

and Steinberg, 1987a). The multiplicity of the antibodies expressed in SLE (human and murine) appears to support the concept of polyclonal B cell activation (Klinman and Steinberg, 1987a). Also repeated exposure to B cell mitogens such as LPS have been shown to lead to the development of antibodies to dsDNA, and immune complex deposition in their kidneys (reviewed in Schwartz, 1993). However the importance of polyclonal B cell activation in the induction of systemic autoimmune disease is questionable since the autoantibodies involved in these diseases are high affinity IgG autoantibodies that exhibit somatic mutations compatible with antigen-driven response, while those elicited by polyclonal B cell activators are low-affinity IgM antibodies. Fifth, B cells could have defective development of tolerance for self-antigens. The development of tolerance for self antigens possibly requires the elimination of autoreactive B cells through clonal deletion (Hartley et al., 1991; Nemazee and Burki, 1989), anergy (Goodnow et al., 1988), receptor editing (Radic et al., 1993) and competition for follicular niches (Cyster et al., 1994). Experiments have shown that failure of mice to undergo negative selection can lead to the expression of autoantibodies. For example, B cells from mice with defective expression of *fas*, or with overexpression of *bcl/2*, have been shown to have antibodies to dsDNA and other nuclear antigens, and to immune-complex nephritis (Strasser et al., 1991). This suggests that autoimmune prone mice have problems at the B cell selection and maturation levels (Strasser et al., 1991; reviewed in Schwartz, 1993). Lastly, similarities have been shown between the shift in specificities that occur in old age and in autoimmunity. This has suggested the possibility of regarding the process of aging as an autoimmune phenomena and vice versa, autoimmunity as accelerated aging (Yoshida et al., 1989; Klinman, 1992). No single explanation can account for the expansion and activation of autoreactive B cell clones, more likely, the breakdown of tolerance is the result of a combination of the

mechanisms described above.

B1 cells: B cells are generally categorized as B1 and B2 Lymphocytes based on their phenotype, origin, and nature of the antibodies. B1 cells constitute about 2 % of the B cells in the spleen of an adult mouse, but are a larger portion of the B cells in the new born. Phenotypically, these cells are IgM^{++} and $IgD^{\pm}$, $B220^{low}$ and may express CD5 (B1a are $CD5^{+}$ and B1b are $CD5^{-}$). These cells are also present mainly in the peritoneal cavity, appear early in ontogeny, and secrete low affinity IgM, IgA and IgG3 antibodies that are polyreactive (reviewed in Murakami and Honjo, 1995). B1 cells also appear to be able to proliferate without T cell help in the presence of natural antigens (reviewed in Hardy et al.,1994). Given their self renewing properties, it has been suggested that B1 cells could play a role in immunological memory (UytdeHaag et al, 1991). Interestingly, these cells seem also to be more prone to produce autoantibodies, and their population is increased in RA and SS patients (reviewed in Tsokos, 1992) and in autoimmune-prone mice (Youinou et al.,1993; reviewed in Hardy et al.,1994). B1 cells specificities include heteroantigens (polysaccharides, lipids and phospholipids such as phosphorylcholine), as well as autoantigens (bromelin-treated erythrocytes (Br-ME), immunoglobulins and ssDNA) (reviewed in Murakami and Honjo, 1995). In mice transgenic for anti-red blood cells (RBC) most conventional B cell (B2 cells) are deleted, but peritoneal B1 cells are spared due to the lack of RBC in peritoneum. These mice develop autoimmune hemolytic anemia and the number of autoantibody producing cells in peritoneal cavity is correlated with the severity of the disease. Elimination of peritoneal B1 cells ameliorates autoimmune hemolytic anemia in these mice (reviewed in Murakami and Honjo, 1995), suggesting that B1 cells are involved in the pathogenesis of this autoimmune disease. Interestingly, only the mice that were not under germ-free conditions developed

autoimmune hemolytic anemia. This reinforces the concept that multiple events have to occur to allow for aberrant recognition of self.

2. Bidirectional interaction of immune, endocrine and central nervous systems

Over the past decade, studies have clearly shown that the immune system interacts with most, if not all, the biological systems to maintain homeostasis. In particular, interactions of the immune system with the nervous and endocrine system have attracted much attention. The study of these interactions has evolved into a new and fascinating field: neuroimmunoendocrinology. These three systems share biochemical pathways and communicate bidirectionally by sharing signal molecules- cytokines, peptide hormones, and neurotransmitters- that act on a set of receptors which are common to these systems. This demonstrates the highly complex regulation of the immune system (Blalock, 1994a).

The notion that there is a correlation between stress and susceptibility to infection, is not new. Ishigami, in 1919 observed that the phagocytic activity of cells from tuberculous patients decreased during episodes of emotional stress. In 1970s, cortisol, a stress-related hormone, was reported to have immunosuppressive effects on antibody-forming cells in rats (reviewed in Brines, 1994; Berkenbosch et al.,1991). At the time the dominant paradigm was that there was a one way flow of information, that is, the central nervous system (CNS) would influence the immune function. It was not until the early 80s that this notion was challenged by observations that an immune cytokine, interferon, was shown to act on CNS and endocrine cells (Blalock, 1994 b), and lymphocytes could

synthesize hormones previously thought restricted to the CNS such as adrenocorticotrophin (ACTH) (Smith and Blalock, 1981). To date, cells from the immune system have been found to produce over 20 neuroendocrine hormones, including growth hormone, prolactin (postulated as IL-2 second messenger), thyrotropin and chorionic gonadotropin (Comprehensively reviewed in Blalock, 1994 a & b). Conversely, cytokines, the immune system mediators, have been shown to act on the CNS and endocrine organs. A clinical example of the integration of the three biologic systems is evident in the systemic response to infection, which include fever, loss of appetite, and even dysmenorrhea (reviewed in Cunningham and De Souza, 1993). The connection of the immune, nervous and endocrine systems is illustrated by inflammatory cytokine IL-1 and IL-6 which are synthesized by a variety of cells including macrophages and thyrotrope cells of the pituitary. These cytokines have been shown to function as hypothalamic-releasing factors. IL-1 induces hypothalamic release of corticotropin releasing hormone (CRH), which in turn leads to pituitary ACTH release, as well as sensitization of the pituitary to direct ACTH-releasing activity by IL-1 (Payne et al., 1994; Besedovsky et al., 1991). IL-1 has also been shown to act directly on the adrenal glands to induce corticoid release which in turn acts on glucocorticoid-response elements, which are associated with various genes encoding for cytokines as well as in other regulatory elements of the inflammatory and immune response (reviewed in Wilkens, 1995). The sites for cytokine actions in the brain are not restricted to the hypothalamus-pituitary-adrenal axis, since receptors for IL-1 have been identified at high density in the hippocampus, the choroid plexus, and the meninges (reviewed in Blalock, 1994 a & b; Marguette et al, 1990). Receptors for IL-1 can also be found on pancreatic islet, where they block the release of insulin and on leydig cells where they inhibit androgen production (reviewed in Cunningham and DeSouza, 1993).

Stated above are just a fraction of the complex interactions between the immune, nervous and endocrine systems. This exciting new information, although fragmentary, strongly suggests new roles for the CNS and the immune system: an immunoregulatory role for the CNS, and perhaps even a sensory role for the immune system (Blalock, 1994b). These findings are already leading to new thoughts in therapy, such as the use of opiate antagonists (which may block lymphocytic endorphins) to prolong allograft survival (Arakawa et al.,1992) or the use of dopamine agonists (to block PRL release) to treat experimental autoimmune encephalitis (EAE) (reviewed in Blalock, 1994b).

3. Gender and sex hormones as modulators of the immune response:

There are marked gender differences in the immune system, and sex hormones are thought to play an important role in the regulation of the immune function.

a. In humans:

Gender differences in the immune response: In humans, women in general have higher levels of immunoglobulins, and mount stronger humoral responses to a variety of antigens than men (reviewed in Ansar Ahmed et al, 1985a; Lahita, 1990, Schuurs and Verheul, 1990; Grossman, 1985; da Silva, 1995). This allows them to respond better to most microbial (e.g. *E.coli*, Brucella, measles, rubella and hepatitis B) and non-microbial antigens than their male counterparts (Ansar Ahmed et al, 1985a; Schuurs and Verheul, 1990; Grossman, 1985; Kenny and Gray, 1971). A special event in hormonal status, pregnancy, is known to downregulate delayed type hypersensitivity (DTH) and allograft rejection, and induce higher immunoglobulin levels (Chaouat, 1993). However,

a detailed description of this phenomena is beyond the scope of this review.

Gender and sex hormones in autoimmune disease: The greater immune responsiveness in women is also evident in their higher susceptibility to autoimmune diseases, especially during their reproductive years. For example, women to men susceptibility ratios for Systemic lupus erythematosus (SLE), Sjogren syndrome (SS), and rheumatoid arthritis (RA) are 10:1, 9:1 and 2-7:1, respectively (reviewed in Ansar Ahmed et al, 1985a; Ansar Ahmed and Talal, 1988; Ansar Ahmed et al, 1993a). Further, 3 cases of otherwise healthy women who received ovulation-induction agents, which induce high levels of estrogen, and developed full-blown SLE have been reported recently (Ben-Chetrit and Ben-Chetrit, 1994). In addition, in autoimmune patients, the course of the disease has been shown to be modulated by changes in their hormonal status such as pregnancy, menses or the use of oral contraceptives (Ansar Ahmed et al, 1985a; Schuurs and Verheul, 1990, Ansar Ahmed and Talal, 1993; Hazes et al., 1990a & b). Female SLE patients have been shown to have an increase in aromatase activity leading to augmented conversion of androgens to estrogens in peripheral tissues (Folomeev et al.,1992). Also, abnormal patterns of estrogen metabolism have been reported in both male and female SLE patients, including an increased 16α-hydroxylation that results in the accumulation of biologically active 16 α-hydroxyesterone and estrol metabolites which are thought to induce a chronic hyperestrogenic state (Lahita et al, 1979; Lahita et al., 1981; reviewed in Ansar Ahmed and Talal, 1993). Pregnancy (a period of high estrogen levels) has been shown to induce flare-ups of SLE either during the course of the pregnancy or postpartum. SLE patients have been shown to have reduced levels of androgens, testosterone, and dehydroepiandrosterone (DHEA) and DHEA-sulfate (Lahita et al., 1987; reviewed in Ansar Ahmed and Talal, 1993 and Lahita, 1990).

Rheumatoid arthritis (RA) is more frequent and also behaves more aggressively in women (reviewed in da Silva, 1993). In RA, the onset of the disease peaks around menopause, while in males the incidence increases with the decline of androgens (Cutolo et al., 1988; reviewed in da Silva, 1993). No consistent differences have been found with regards to levels of estrogen or testosterone in sera from RA patients (da Silva, 1993); there are, however, reports on reduced levels of dehydroepiandrosterone (DHEA) (Da Silva, 1993). Hormonal level fluctuations during menstrual cycle, or due to consumption of oral contraceptives have had variable effects on RA patients (Hazes et al.,1990; Hernandez-Avila et al., 1990; van Zeben et al.,1990). Pregnancy has been found, in most studies, to induce a remission of RA (Ostensen et al.,1983). The above observations suggest that sex hormones may be involved in the pathogenesis of autoimmune diseases.

Hormonal drugs and the immune function: The use of hormones in the form of oral contraceptives and postmenopausal hormonal replacement is widespread, and often sustained for many years. Long term estrogen administration has been associated with breast and endometrial cancer (Mack and Ross, 1989). Reports on the effects of oral contraceptives on the immune system have shown often conflicting results. Patients taking oral contraceptives have been shown to have normal levels of immunoglobulins (Bisset and Griffin, 1988); however, the antibody response to tetanus toxoid *in vivo* was reduced (Joshi et al., 1971). No significant increase in the expression of autoantibodies has been reported (Keller et al., 1971). Reports on the proliferative response of lymphocytes from patients taking oral contraceptives to mitogens such as phytohemagglutinin (PHA) or concanavalin A (Con A) show either no change (Dweyer et al., 1975; Mori et al., 1975; Gerretsen et al., 1980; Tezabwala et al., 1983) or a

significant decline in the response (Keller et al., 1977; Barnes et al., 1974). The Royal College of General practitioners found an increase in viral infections such as varicella in women taking oral contraceptives (reviewed in Keller, 1976). There is a wide diversity in the chemical composition of oral contraceptives available, and this probably accounts for some of the differences observed. Women taking oral contraceptives with estrogenic activity had reduced cell-mediated immune responses and enhanced antibody production which was not present in women taking oral contraceptives with a higher progesterone component (Gerretsen et al., 1978). In women taking estrogen and progesterone substitution for primary ovarian failure, PHA-induced lymphocyte proliferation was also depressed (Morishima and Henrich, 1974). In males treated with estrogens after prostatic cancer, the splenocyte proliferation to PHA was also reduced, when compared to their own reactivity prior to the hormonal treatment (Ablin et al., 1974).

Hormonal contraception and the immune function in autoimmune patients: Hormonal contraception in autoimmune patients has been a matter of controversy. Systemic lupus erythematosus has a predilection for young women of reproductive age. Most authors agree that oral contraceptives in SLE patients are not indicated since they can activate the disease (Jungers et al.,1982; Jungers et al., 1990). SLE patients that take oral contraceptives have been found to have increased risk of deep venous thrombosis (Julkunen et al., 1993). Contrary to what has been observed in lupus patients, oral contraceptives have not been associated with worsening of RA (reviewed in Schuurs and Verheul., 1990). Further, it has been proposed that women taking oral contraceptives have lower incidence and less severe RA (Vandenbroucke et al., 1982; van Zeben et al., 1990; Hazes et al.,1990); however, not all studies found a reduction in the incidence of RA (Hernandez-Avila et al., 1990). Again, the varying chemical composition of estrogen

and other hormones in oral contraceptives could explain for inconsistent findings. Recent studies have explored to possible therapeutic value of oral contraceptives (Van Den Brink and Bijlsma, 1989 Hall et al., 1994).

b. In murine models of autoimmune diseases:

Analogous to the human situation, gender differences in immune capabilities have also been noted in a number of experimental laboratory animals (reviewed in Ansar Ahmed et al, 1985a; Grossman, 1985; Schuurs and Verheul, 1990; Ansar Ahmed and Talal, 1990). These observations suggest a possible influence of gender and sex hormones in the humoral and cell-mediated immunities.

Gender and sex hormones in nonautoimmune strains: Female mice have been reported to have higher total immunoglobulin levels, mount a stronger response to vaccination, and produce more antibodies to a variety of hetero- and self- antigens compared to males (Ansar Ahmed et al.,1989b; Ansar Ahmed and Talal, 1990). Reports on the cell-mediated immune capabilities of male and female mice are more diverse. Female laboratory animals have been reported to have reduced incidence of tumors and reject allografts more rapidly than males (Graff, 1969; reviewed in Ansar Ahmed and Talal, 1990; Ansar Ahmed et al.,1985a). Further, gonadectomy, adrenalectomy or the combination of both, resulted in accelerated rejection of allografts in males, while oophorectomized females that received transplanted testis had showed a significant reduction in their ability to reject the implant (Graff et al, 1969). On the other hand, the

immune response to intracellular parasitic infection with *Schistosoma mansoni* was stronger in males than in female CBA/J mice (Eloi-Santos et al., 1992). Also, castrated C57BL/6J, C3H, and DBA/1 mice that were treated with estrogen had reduced delayed type hypersensitivity with increased levels of antibodies (Myers and Petersen, 1985; Calsten et al., 1989). Other sex hormones, such as DHEA have also been shown to modify the susceptibility to viral infections (Coxsackie virus B4 and herpex simplex type 2) (Loria et al., 1988).

Gender and sex hormones in experimental models of autoimmunity: The effects of sex steroids have been observed in several experimental animal models for autoimmune diseases (Reviewed in Ansar Ahmed et al.,1985a; Ansar Ahmed and Talal, 1990; Ansar Ahmed and Talal, 1993). Female NZB x NZW F_1 (B/W) mice, as an experimental model for SLE, spontaneously develop autoantibodies, lymphadenopathy, arthritis, immune complex glomerulonephritis, and die earlier than their male counterparts. Prepubertal castration (with corresponding drop in testosterone levels) of the B/W males cause premature death, suggesting a protective effect for the male hormones (Roubinian et al., 1977; Siiteri et al.,1980). Prepubertal castration of females did not change their mortality. In these mice, however, administration of androgens to castrated females reduced the anti-DNA antibody level and prolonged their life span (Roubinian et al.,1977; Siiteri et al., 1980; Melez et al., 1980). Importantly, the protective effect of androgens could be attained even when administered after the onset of diseases (Roubinian et al., 1979; Melez et al.,1980). The modulation of the course of the disease by castration and sex hormones was negated by concomitant thymectomy, suggesting that the thymus is the site of hormonal activity that influences the immune function (Roubinian et al, 1978). Administration of estrogen to B/W males resulted in an

accelerated development of severe nephritis accompanied by higher levels of anti-DNA antibodies and enhanced mortality (Roubinian et al.,1978). Subsequent studies showed that dihydrotestosterone (DHT)-treated mice had reduced levels of anti-DNA antibodies and improved the survival of both, male and female, B/W mice while administration of estrogen had a deteriorating effect (Roubinian et al., 1978; Roubinian et al., 1979). Treatment with ethinyl-estradiol of NZB/W mice has also been shown to increase the incidence of cancer, mainly originating in the lymphoid organs (Keisler et al., 1991). Another animal model for SLE, MRL//Mp-lpr/lpr (MRL/lpr) mice, which are characterized by development of lymphadenopathy and splenomegaly, expression of autoantibodies (against dsDNA, cardiolipin, and Sm), antigen-antibody deposition in the kidney, and glomerulonephritis that resembles that of SLE patients. In these mice, differences between male and female with regards to the time of expression of autoantibodies and development of the disease were less remarkable. However, MRL/lpr mice that were treated with physiologic doses of 17β-estradiol had an accelerated development of autoantibodies, lymphadenopathy, glomerulonephritis and death (but reduced T-cell mediated lymphocytic infiltration in the submandibular glands and perivascular lesions in kidneys) (Carlsten et al.,1990; Carlsten et al., 1991). Androgen treatment of these mice led to reduced autoantibodies to DNA and prolonged survival (Steinberg et al.,1980) In non-spontaneous models for SLE, such as those induced in nonautoimmune BALB/c mice by administration of human anti-DNA (16/6 Id+) antibodies (Blank et al., 1990), estrogen treatment was shown to accelerate the disease. In contrast, androgens and estrogen antagonists such as tamoxifen, delayed it (Sthoeger et al.,1994) . In other models of SLE, including NZB x SJL/J, NZB x CBA, NZB x C3H, NZB x DBA/2, androgens and an estrogen antagonist (tamoxifen) have been shown to retard, while estrogens accelerate their expression of autoimmune disease and mortality

(reviewed in Ansar Ahmed and Talal, 1990). Another murine model for SLE, the BXSB/Mp mice, is characterized by a worse disease and higher mortality in males than in females. The acceleration of the expression of the disease in males is dependent on a Y chromosome-linked gene (yaa) (Fossati et al., 1995; reviewed in Hahn, 1993).

Sex hormone regulation of autoimmune reactivity has also been noticed in animal models for other autoimmune diseases: In DBA-1 mice, a model for induced collagen arthritis, and in B10.RIII mice which develop a chronic experimental autoimmune encephalomyelitis, treatment with 17β-estradiol has been shown to delay the onset, and reduce the severity of the disease (Jansson et al., 1990; Jansson et al.,1994). In NOD mice, which are prone to Insulin Dependent Diabetes Mellitus (Yagi et al.,1991), the expression of the disease is accelerated in the females; similar results have been obtained in Lewis rats (LEW/N rats), used as a model for polyarthritis; or PVG/c rats which develop autoimmune thyroiditis (reviewed in Ansar Ahmed and Talal, 1990).

Other hormones that have been shown to have important modulatory effects on the immune system and possibly play a role in the development of autoimmune diseases include: prolactin (reviewed in Chikanza and Panayi, 1991; Buskila et al.,1992; Jara et al.,1991); dehydroepiandrosterone (DHEA)(reviewed in Wilder, 1995); Growth hormone (GH) (Murphy et al.,1992); progesterone (Fincl and Ciaccio, 1980). In recent years, their role as modulators of the immune function has begun to be unraveled. A full description, however, is beyond the scope of this review.

c. In other animals:

The information available on the effects of sex steroids on the immune function of companion or farm animals is scant. In dairy cows the IgG levels in sera have been shown to decrease at estrous and at calving, and this decline was correlated with reduced serum progestrone levels. A similar profile was found *in utero*, with high levels of IgG during the luteal phase but undetectable in estrous (Brenner et al.,1995). Peripheral lymphocytes from beef steers treated with estrogen/progesterone implants showed an increase in the proliferative response to mitogens such as Con A and PHA or to heteroantigens (OVA) (Burton et al.,1993). Gender differences have also been observed in fish and several reptiles (reviewed in Zapata et al., 1992).

d. Age of exposure to estrogens:

The effects of exposure to estrogen are more evident early in life. In mice, female fetuses have been found to have higher levels of 17β-estradiol in sera and amniotic liquid. Intrauterine hormonal environmental differences as small as those resulting from the gender of the neighboring pups *in utero* have been shown to influence the development of genitalia, aggressiveness, sexual behavior in the adult and aggressiveness (von Saal et al., 1983; vom Saal et al.,1990). The effects of early exposure to estrogen on the immune system of mice are important, and can often be deleterious. For example, administration of estrogen to pregnant C57BL/6J mice induced an earlier expression of Sjögren-like lesions in the salivary gland of the progeny (Ansar Ahmed et al., 1989a). Prenatal exposure of (NZB/NZW) F1 mice to testosterone prolonged the life spans of male offspring, while the females of the same litter remained unaffected (Keisler et al., 1991). In these mice, administration of small doses of testosterone early in life was the most effective time to delay the expression of autoantibodies and the development of the

disease (Melez et al., 1980). Neonatal administration of estrogen has been shown to induce profound thymic atrophy, depletion of Thy 1.2^+ cells, reduced $CD8^+$ cells, and delayed DTH (reviewed in Ansar Ahmed and Talal, 1993).

In humans, prenatal exposure to a synthetic estrogen, diethylstilbestrol (DES), which was given to pregnant women to reduce nausea and help pregnancies to term, lead to an increased incidence of cancer and autoimmune disease (Noller et al.,1988; Herbst, 1981; Holladay et al.,1993; Ways et al. 1987).

4. Overview of the specific effects of female gender and estrogen:

a. Effects on B cells

In humans, women (especially of ages 14-19) have significantly higher levels of IgM immunoglobulins than males of the same age. This difference is not apparent after menopause (Butterworth et al., 1967; reviewed in Paavonen, 1987). This suggests sex hormonal influence on immunoglobulin levels. Plasma immunoglobulin levels, however, were not altered in women taking oral contraceptives (Bisset and Griffin, 1988). *In vitro*, however, lymphocytes cultured in the presence of 17β-estradiol did not modulate proliferation alone, but did magnify the proliferative response to pokeweed mitogen (PWM) (Sthoeger et al.,1988; Kalman et al.,1989; Paavonen et al., 1981), or sheep red blood cells (SRBC) (Clerici et al.1991). Estrogen has been reported to act together with PWM to increase the number of IgM secreting cells (Paavonen et al., 1981). Others have reported a preferential increase in IgG and IgA producing B cells (Kalman et al., 1989). Interestingly, the effects of estrogen were independent of the hormonal status of the

lymphocyte donor (male, female, luteal or estrous phase of menstrual cycle, or even menopause) (Sthoeger et al.,1988; Clerici et al., 1991). The estrogen enhancing effect on PWM-induced proliferation could be replicated by preincubating the cells with estrogen and subsequently stimulating them with PWM. However, this enhancing effect was not observed when cells were first exposed to PWM and then to estrogen. These data indicate that the potentiating effect of estrogen on lymphocytes in culture occurs during the first 12 hours *in vitro* (Sthoeger et al.,1988). The reported effects of co-culture of PWM-stimulated lymphocytes with testosterone ranged from no effect (Clerici et al., 1991) to suppression of generation of PFC (Sthoeger et al.,1988).

In mice, normal females have higher immunoglobulin levels and mount higher antibody responses to a variety of microorganisms than males (reviewed in Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990; Carlsten et al.,1989). Estrogen, has been shown to regulate the expression of serum and uterine immunoglobulins (Lahita, 1990; Prabhala and Wira, 1995) and to augment the antibody production to a variety of hetero- (reviewed in Ansar Ahmed, 1985a; Paavonen, 1987; Schuurs and Verheul, 1990) and self- antigens such as bromelain-treated erythrocytes (Br-ME) or oxazolone (Ansar Ahmed, et al., 1989a; Ansar Ahmed et al., 1989b; Carlsten et al.,1989). In nonautoimmune mice, estrogen has been shown to accelerate the expression of total and specific antibodies in C3H, C57BL/6J, but not in BALB/c, DBA/1 or NFR/N mice (Nilsson and Carlsten, 1994; Calsten et al., 1989). In Sprague Dawley rats, estrogen was shown to increase the level of IgM antibodies without concomitant increase in plaque-forming-cells (Myers and Petersen, 1985). Higher reactivity of the humoral immune system of normal female C57BL/6J, NZW, and C3H/HeJ, with increased levels of antibodies to Br-ME, presumably produced by B1 ($CD5^+$ B cells) cells, compared to

their male counterparts (Ansar Ahmed et al., 1989b). Further, administration of estrogen but not testosterone induced an increase in the activity of these autoantibody forming cells (Ansar Ahmed et al., 1989b). The increase in plaque forming cells (PFC) to Br-ME observed in C57BL/6J mice appeared to be due to a shift in the specificity of B1 cells, and not due to an increase in the total numbers of these cells.

In autoimmune mice, NZB/W F1, MRL +/+ and MRL/lpr females have been shown to have higher levels of autoantibodies than their male counterparts (Cohen et al.,1988). Estrogen has been shown to accelerate the expression of autoantibodies and hasten death of MRL/lpr and NZB x NZW F_1 (B/W) mice (Brick et al., 1985; Siiteri et al., 1980; Roubinian et al., 1978; Carlsten et al., 1989; Carlsten et al., 1992; reviewed in Ansar Ahmed and Talal, 1993). Similarly, in nonautoimmune BALB/c mice injected with 16/6 id+ antibodies, estrogen treatment accelerated the development of autoantibodies (Blank et al., 1990).

Estrogen and B cell lymphopoiesis: Bone marrow is the principal site of B lymphopoiesis in mammals. Recently, it has been proposed that estrogen induces a decrease of IL-7 dependent B cell precursors at the bone marrow level (Kincade et al.,1994; Masuzawa et al.,1994). In a recent report, estrogen was shown not to induce death of IL-7 dependent B cell precursors, but to affect lymphopoiesis by altering the bone marrow microenvironment (Smithson et al., 1995). Ovariectomy in mice has been reported to induce an increase in bone marrow cellularity, which was mainly attributable to accumulation of B cell precursors (Masuzawa et al., 1994). Similar results were obtained using genetically hypogonadal female (HPG/Bm-hpg/hpg) mice (Smithson et al.,1994). Estrogen treatment of the hypogonadal mice returned the B cell precursor

numbers to normal. During pregnancy of normal mice, the number of B cell precursors have been shown to be reduced. Further, these results were reported to be mimicked by giving mice a single dose of water-soluble 17β-estradiol (Medina and Kincade, 1994). It should be noted, however, that these effects were only accomplished when very high doses (2.5 mg, but not 0.01 mg) of 17β-estradiol were administered. Estrogen is known to play a key role in the maintenance of the bone mass and to modulate the function of stromal cells in the bone (reviewed in Murad and Kuret, 1990). Further, a number of bone marrow stromal cells including osteoblasts (Eriksen et al., 1988) are thought to have receptors for estrogen, and ovariectomy has been shown to modulate the stromal cell composition (reviewed in Kincade et al., 1994). Estrogen has been shown to inhibit IL-1 synthesis by stromal cells and macrophages (Hu et al., 1988), and to suppress IL-6 production by bone marrow stromal cells, both are powerful mediators of osteoclastic bone resorption (Jilka et al., 1992; Passeri et al., 1993). Also, 17β-estradiol has been shown to increase the production of TGF-β by osteoblast-like cells (Finkelman et al., 1992). TGF-β has previously been shown to inhibit IL-7 dependent B cell development (Lee et al., 1987).

b. Effects on T cells:

Sex steroid hormones have been consistently demonstrated to modulate the T cell function. Estrogen has been shown to delay graft rejection (Graff, 1969), and to reduced delayed type hypersensitivity (Holmdahl, 1989; Carlsten et al.,1989). In a human study, the total number of lymphocytes in women decreased with age and menopause (Giglio et al., 1994). Post menopausal women had reduced numbers of T cells (with concomitant increase in the proportion of B cell), and a relative decrease in $CD4^+$ and

a corresponding increase in $CD8^+$ and NK cells (Giglio et al., 1994). Increases in total number of lymphocytes, B cells, NK cells, and $CD4^+$ cell numbers correlated with LH serum levels, while reduced levels of B cells and CD8 cells correlated with higher FSH and estrogen levels (Giglio et al., 1994). Reports regarding the effects of oral contraceptives on T cell function are inconsistent (see section on gender and sex hormones as modulators of the immune response).

In vitro, culture of splenic T cells with estrogen reduced their subsequent response to PHA (Tezabwala, 1983; Mendelsohn et al.,1977). However, others (Arthreya et al., 1993) have reported that neither estrogen nor testosterone added to peripheral blood mononuclear cells (PMBC) in culture, induced an increase in proliferation to PHA, anti-CD3 or IL-2. Similar results were reported in proliferation studies in response to Con A (Yron et al., 1991). Studies on the effects of sex hormones on T cell subpopulations, however, showed that estrogen reduced the percentage of $CD4^+$ and increased that of $CD8^+$ T cells in response to PHA, while testosterone increased the proportion of $CD4^+$ T cells following IL-2 stimulation (Arthreya et al., 1993). Addition of estrogen to Con A- stimulated peripheral blood lymphocytes induced a marked increase in a subpopulation of T cells presumed to be T suppressor cells (identified by their ability to spontaneously form rosettes in the presence of autologous red blood cells) (Yron et al., 1991). The differential effect of estrogen on lymphocyte subsets could potentially affect the Th1/Th2 balance (Huber and Pfaeffle, 1994). Other sex hormones, such as DHEA, $1,25(OH)_2$ vitamin D, have been shown to modulate IL-2 production by T helper cells (Daynes et al.,1990) and may be important in the regulation of the Th1/Th2 balance (reviewed in Rook et al., 1994).

Sex hormones may modulate T cell function at various levels. Sex hormones have been shown to act on the thymus, as early as 1898 (by Cazolari and Hammar, reviewed in Ansar Ahmed and Talal, 1993). In normal animals, administration of estrogen induces marked atrophy of the thymus (e.g. by inducing apoptosis of double positive thymocytes), while prepubertal gonadectomy leads to thymic hyperplasia (reviewed in Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1993; Ansar Ahmed and Talal, 1990). There is ample evidence that sex hormones act at the thymic levels on the T cell development and maturation (reviewed in Ansar Ahmed and Talal, 1993a). Estrogen receptors have been found in the thymic epithelial/stromal cells (Danel et al., 1983; Haruki et al., 1983). High affinity androgen receptors have been shown in human and murine early thymocytes (Kovacs and Olsen, 1987; Viselli et al.,1995). Sex hormones have been shown to alter the thymic subsets: Castration of C57BL/6J male mice lead to increased proliferative response of thymocytes to Con A, and a reduction in $CD8^+$ T cell subpopulation. Testosterone replacement in these mice lead to reduced numbers of double positive cells and diminished spontaneous proliferation of thymocytes (Olsen et al.,1991). There have been reports that estrogen, but not testosterone, reduces double positive thymocytes (Screpanti et al., 1989); differences in the age of the mice treated (adult vs prepubertal) could account for some of the differences. On mature lymphocytes, high affinity receptors for estrogen have also been identified in mature $CD8^+$ T cells (Cohen et al., 1983).

Estrogen was reported to induce stimulation of extrathymic T cell (Hirahara et al.,1994; Kimura, 1994). In recent years the role of extrathymic T cells (hepatic, and intraepithelial lymphocytes) has been explored. These cells are large granular lymphocytes, express $\tau\delta$ or $\alpha\beta$ TcR, contain forbidden self-reactive clones and expand

with aging. These extrathymic T cells are more predominant in females and are induced further by treatment with estrogen (Okuyama, 1992; Hirahara et al., 1994).

Overall, estrogen has been shown to depress the splenic T cell response to a variety of stimuli (Schuurs and Verheul, 1990; Grossman, 1985; Ansar Ahmed and Talal, 1993; Carlsten et al.,1989.

c. Effects on macrophages:

Phagocytes are also affected by estrogen (Ansar Ahmed and Talal, 1993). Estrogen receptors are present in macrophages (Cutolo et al.,1993). Secretion of IL-1 from peritoneal macrophages was higher in female than male rats. Further, ovariectomy reduced, while estrogen treatment enhanced, the secretion of IL-1 by macrophages (Hu et al.,1988). Estrogen has also been shown to modulate macrophage production of reactive oxygen intermediates and nitrite release (Tzu-Chieh et al.,1994). Importantly, the expression of class II on macrophages appears to be regulated by estrogen as well (Ansar Ahmed and Talal, 1993). B cell response to T cell-independent antigens has been shown to be modulated by macrophage cytokines (reviewed in DeFranco, 1993). It is possible that the estrogen-induced increase of IL-1 secretion could augment the B cell response to T cell-independent antigens such as LPS.

d. Effect on NK cells:

In mice, treatment with estrogens lead to stimulation of NK cell activity during the first month, but prolonged exposure reduced NK cell activity, both *in vivo* and *in vitro* (Seaman et al., 1978; Screpanti et al., 1987; Paavonen, 1987). In mice, the degree of estrogen-induced suppression varied for different strains: high (over 50%) in C3H/N, DBA/1 and NZB/W; and lower (under 30%) in MRL/lpr and C57BL/6J mice (Screpanti et al., 1987). In a recent report, tamoxifen was found to enhance while estradiol inhibited NK cytotoxicity (Baral et al., 1995). Downregulation of NK cell activity may lead to reduced secretion of γIFN, and thus indirectly modulate B cell function

4. Autoimmune diseases and autoantibodies:

a. Definition of autoimmunity and autoimmune diseases:

Autoimmunity is traditionally defined as the body's immune system reacting against itself (reviewed in Schwartz, 1993). The concept was first described by Paul Erlich in the early 1900's as *horror autotoxicus*. We know now that healthy individuals have self reacting lymphocytes (both T and B) which appear to be important in: (i) the clearance of degraded autoantigens and senescent cells; (ii) prevention of autoimmunization with self antigens; (iii) non-specific prompt binding and/or elimination of crossreactive foreign antigens until a specific response can be elicited; and (iv) serving as precursor of clones with higher avidity and affinity for heteroantigens.

Although the potential for autoimmune disease is normally present, a number of

mechanisms are in place to prevent it. In several clinical situations including infections, intake of drugs, or even in aging this tight regulation can be skewed and allow for signs of autoimmune disease to appear. In addition, autoimmune mechanisms are thought to underlie in a wide range of clinical diseases that are not traditionally considered immunologic in nature ranging such as certain types of epilepsy (Chikladze et al., 1994).

Autoimmune diseases can be classified into organ-specific and systemic diseases. Systemic autoimmune diseases represent a worldwide, major health problem, affecting about 2% of the population. Although the mode of expression, severity and progression of these diseases are quite variable, the long term prognosis in terms of suffering and disability as well as financial burden are serious.

Systemic autoimmune diseases are characterized by their diverse clinical manifestations, so much so, that their diagnosis is based on compliance with a set of multiple criteria defined by the Arthritis and Rheumatism Association (ARA). This has lead to the hypothesis that diseases such as SLE or RA may encompass multiple diseases. One of the reasons of this heterogeneity is probably that systemic autoimmune diseases are multifactorial in origin, with genetic, endocrine, infectious, and environmental elements contributing to their development. Ensuing are brief descriptions of the most salient features of some of the diseases that are relevant to the present work:

b. Autoimmune diseases:

Systemic lupus erythematosus: This disease, which has a prevalence of 1.5 to 5 per million in the USA, is characterized by arthralgias, myalgias and non-erosive

polyarthritis of the joints in extremities; cutaneous lesions (malar rash, ulcers and photosensitivity); cardiovascular abnormalities (pleurisy, pericarditis) and renal defects (glomerulonephritis). Anemia, leukopenia, lymphopenia, psychosis and seizures, fatigue, malaise, fever and weight loss are also known to occur. Fertility rates are normal, but spontaneous abortions and stillbirth rates are high, and pregnancy often leads to disease flare-ups during the first trimester or post partum (Gimovsky et al., 1984). The production of autoantibodies to a wide range of self antigens (dsDNA, cardiolipin, nuclear antigens, Sm, RNP, Ro, La, etc.) and deposition of immune complex leading to renal failure are important features of SLE. Currently there is no cure for SLE. Patients are managed with glucocorticoids, and nonsteroidal antiinflammatory drugs. A number of antiestrogens and androgens including Danazol, 19-Nortestosterone, Nandrolone-decanoate (19-testosterone) progestagens, and antigonadotropic agents (cytoproterone) have been proposed to treat SLE. To date, these drugs have not yielded consistent beneficial effects (reviewed in Lahita, 1993; Ansar Ahmed and Talal, 1988; Van Vollerhoven and McGuire, 1994).

Rheumatoid Arthritis: A chronic, systemic, inflammatory condition characterized by symmetric polyarthropathy with progressive destruction of cartilage, and bone erosion that leads to deformity and disability. Two thirds of the patients have rheumatoid factor, an autoantibody to the patient's own IgG in their sera. Overactive T cells in the synovium are thought to play a central role in the self-perpetuating inflammation that characterizes this disease. Sex hormones are thought to play an important role in RA. Signs and symptoms have been found to change with the menstrual cycle, with improvements during the luteal phase, and there have been reports of improvement with the use of estrogen-based oral contraceptives, and even disease regression during

pregnancy (reviewed in da Silva, 1993). Hormonal replacement therapy in post menopausal women with RA elicited only marginal clinical improvement (Hall et al., 1994) Thorough studies with regard to the potential beneficial effects of estrogens given for long term treatment are needed. Therapy for RA remains empirical: patients are usually managed with nonsteroidal anti-inflammatories, glucocorticoids and other compound including gold, D penicilamine and antimalarials.

Sjögren Syndrome: An autoimmune disease characterized by the progressive destruction of salivary and lacrimal glands leading to mucosal and lacrimal dryness. The salivary glands have lymphocytic infiltration and immune complex deposition. In addition, 10% of the patients develop lymphoproliferative processes. Treatment is symptomatic and immunosuppressive agents are of value.

Antiphospholipid Syndrome: This autoimmune disease is characterized by the expression of antibodies to cardiolipin and other phospholipids. Clinical presentation includes arterial and venous thrombosis, thrombocytopenia, recurrent fetal loss and low birth weight, cardiac and neurologic manifestations, and/or livedo reticularis (Sammaritano and Gharavi, 1992; Lockshin, 1994).

6. Review on estrogen:

a. Physiology of estrogens:

Ovaries are the principal source of estrogen in premenopausal women. The major secretory product is estradiol, synthesized by the granulosa cells from androgenic

precursors provided by the thecal cells. Secreted estradiol is oxidized reversibly to estrone and both these compounds can be converted into estriol. All three can be detected in urine. In men and post menopausal women, the main source of estrogen is the adipose tissue, where estrone is synthesized from adrenal dehydroepiandrosterone. During pregnancy the main source of estrogen is the placenta which utilizes fetal dehydroepiandrosterone and 16α-dehydroepiandrosterone to produce estrone and estriol (reviewed in Murad and Kuret, 1990).

Estrogens act on a variety of target organs: Besides being instrumental in the menstrual cycle and in the development of sexual secondary characters, estrogens act as weak anabolic factors in the liver, and modulate lipid metabolism (reviewed in Murad and Kuret, 1990). Estrogens act on their target tissues by diffusing passively through the cell membrane into the cytoplasm, and bind to a receptor that is translocated into the nucleus. In the nucleus, the activated ligand binds to a specific DNA sequence, the hormone-responsive element. Like other steroids, estrogens act by regulating the transcription of certain genes. Alternatively, another theory states that estrogen may diffuse unattached into the nucleus where it binds to the receptor and acts on the transcription of certain genes (reviewed in Murad and Kuret, 1990).

b. Sources of exposure to estrogen:

In the females, hormonal levels are not constant, rather they are subjected to regulated fluctuations throughout life. These changes can be physiologic such as those occurring in menarche, menses, pregnancy, or menopause. Under certain circumstances, however, non-physiologic levels of sex hormones may be present. These hormones may be

endogenous (eg: ovarian or adrenal cancer) or exogenous. There are several ways by which both humans and animals are exposed to exogenous estrogens: (i) Therapeutic. A large percentage of the female human population uses estrogen-based oral contraceptives. In the USA, the prevalence of all women ages 15 to 45 taking oral contraceptives ranged between 1965 and 1984 from 20 to 30%. In younger women (under 24 years old), the prevalence is much higher, 55 to 65% (Annegers, 1989). Similar figures are reported in Britain (Vessey and Thorogood, 1989). Further, in recent years estrogen replacement for post menopausal (natural or surgical) women has become a routine clinical practice. Also the use of estrogen-based drugs prescribed to patient with dysmenorrhea, endometriosis, and fertility problems, etc., is growing steadily (Murad and Kuret, 1990) (For full list of estrogen indications see **table 2.1**). (ii) Nonsteroidal compounds with estrogenic activity occur naturally in a variety of plants including flavone, isoflavone and other phytoestrogens. Plant products, such as soybean have recently been shown to have isoflavonic estrogens that can actively modify the endocrine status (Moltoni A. et al., 1995; discussed in Goldin, 1994). In mice, dietary soybean has been shown to induce the growth of uteruses and to reduce prostatic growth in immature mice (Makela S.I. et al., 1995). (iii) It is now recognized that a number of chemicals, such as 2,4-D, dioxin, organochlorins, DDT, among others., that are released into the environment have been shown to bind to intracellular steroid hormone receptors and evoke hormonal effects in animals and humans (Soto et al., 1994; Gray et al., 1994; reviewed in Colburn T. et al.1993). These compounds have been collectively termed endocrine disruptors.

c. The levels of estrogen in mice:

Although in most studies estrogen levels were assessed by RIA, there are discrepancies

in the current literature regarding the physiologic levels of sex hormones in mice. This is thought to be due a lack of a unified assay protocol and adequate controls. **Table 2.2** briefly lists some of the concentrations reported in the literature.

Table 2.1 : FDA approved uses of oral contraceptives

Oral contraceptives
Menopause
Dysmenorrhea
Dysfunctional uterine bleeding
Failure of ovarian development
Acne
Hirsutism
Prevention of Heart attack
Osteoporosis
Prostatic carcinoma
Suppresion of postpartum lactation

(Extracted from Murad and Kuret, 1990)

Table 2.2: Murine levels of estrogens reported in the literature

STRAIN	ESTROGEN LEVELS	METHOD	REFERENCE:
Balb/c	♂ <40 ; ♀ <40 pg/ml	RIA	Brunner et al..1986
C57BL/6J	♂ 67.9 pg/ml ♀ 345.7 pg/ml	RIA	Ansar Ahmed et al..1989b
NZB/W DBA/2	♀ 21 pg/ml ♀ 30 pg/ml	RIA	Brick et al.. 1985
DBA/1	♀ <12 pmol/l	RIA	Jansson et al.. 1990
MRL/lpr C57BL/6-lpr	♀ <300 pmol/l ♀ <300 pmol/l	ELISA	Carlsten et al.. 1989
ddY	♀ 17.8 pg/ml	RIA	Masuzawa et al.. 1994

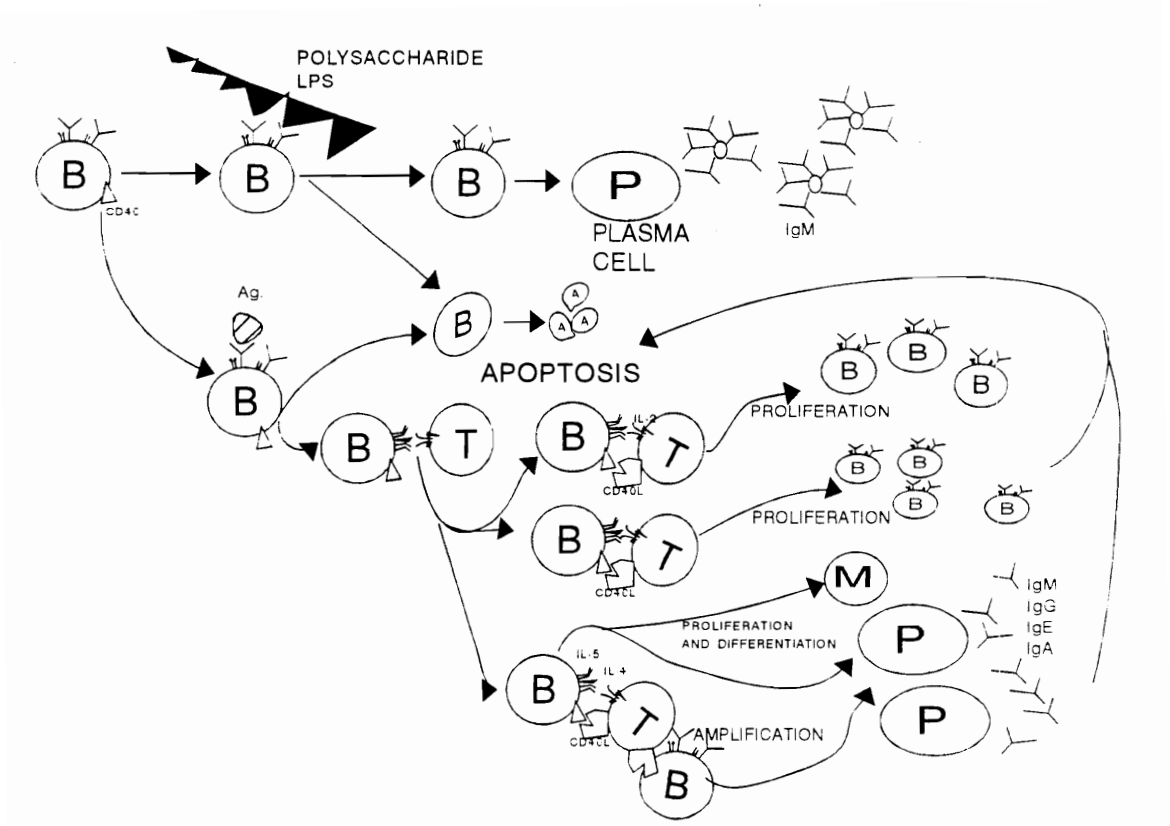

Figure 2.1: B cell activation by T cell independent and T cell dependent antigens. The fate of the B cell is determined by the type of antigen and by the combination of cytokines present: (i) B cells may divide and differentiate into antibody-producing cells (plasma cell). This is largely dependent on the presence of Th2-type cytokines: IL-4, IL-5, IL-6; Hodkin et al.,1990; Noelle et al., 1991). (ii) In the presence of IL-2 (mainly secreted by Th1 cells), however, B cells proliferate, without significant differentiation into plasma cells (Noelle et al.,1991; Hodgkin et al., 1991; Hodgkin and Kehry, 1992). Other cytokines such as γIFN (Th1) or TGFβ (Th3 ?) are known to have an inhibitory effect on B cell proliferation (Kanof et al., 1991; Massagué, 1990).

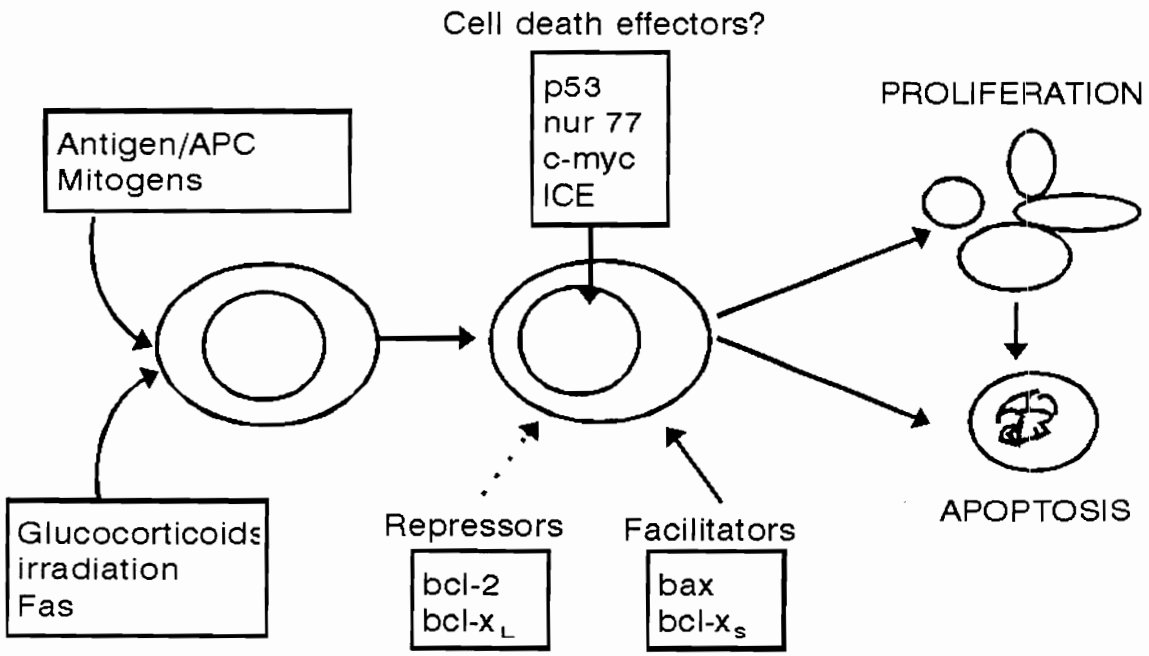

Figure 2.2: Genetic regulation of apoptosis.

CHAPTER 3:

ANTIBODIES TO CARDIOLIPIN IN NORMAL MICE: INDUCTION BY ESTROGEN BUT NOT DIHYDROTESTOSTERONE

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ABSTRACT

Autoantibodies against cardiolipin, a phospholipid, have been demonstrated in a variety of pathological states including several autoimmune conditions in humans and in certain lupus-prone mice. In this study we detected antibodies reactive to cardiolipin in normal C57BL/6J mice by ELISA. These autoantibodies are detected less frequently in the serum of male than in female mice thereby suggesting the influence of sex hormones. The relative refractoriness of normal male mice to the induction of anticardiolipin antibodies was not due to the suppressive effects of male hormones, since prepubertal orchiectomy with or without treatment with 5α-dihydrotestosterone had little influence on the expression of these autoantibodies. Administration of estrogen, however, induced a marked increase in the incidence as well as the levels of these autoantibodies in male and female mice regardless of the presence or absence of gonads. The effect of estrogen was not restricted to C57BL/6 mice, since nonautoimmune BALB/c female mice also developed these autoantibodies. Estrogen-treated mice also had varying levels of antibodies reactive against other membrane phospholipids including anionic phospholipids, phosphatidylinositol (PI), phosphatidylserine (PS), and zwitterionic phospholipids, phosphatidylcholine (PC), but not phosphatidylethanolamine (PE). Antibodies to cardiolipin were found to crossreact with anionic but not zwitterionic phospholipids. To our knowledge, this is the first report on the demonstration of antiphospholipid autoantibodies in normal mice and induction of these antibodies by estrogen. The estrogen-induced expression of antibodies to cardiolipin was also evident in BALB/c mice. In addition we report that the expression of antibodies to cardiolipin in autoimmune-prone BXSB mice is associated with the Yaa gene. BXSB -$^{+yaa}$ have a higher incidence and levels of antibodies to cardiolipin than the BXSB mice that lack the *yaa* gene.

INTRODUCTION:

Autoantibodies against membrane phospholipids such as cardiolipin have been detected in a subset of patients with systemic lupus erythematosus (SLE) (reviewed in Mackworth-Young, 1990; Sturfelt et al., 1987) and primary Sjögren's Syndrome (Pennec et al., 1991). These autoantibodies are frequently detected in patients with antiphospholipid syndrome (APS) (reviewed in Parke, 1989; Harris, 1990) who manifest diverse clinical conditions such as arterial and venous thrombosis of large vessels, thrombocytopenia and repeated fetal loss. In general, moderate to high levels of IgG rather than IgM anticardiolipin antibodies have been closely associated with thrombocytopenia, fetal distress and/or fetal loss (Harris et al., 1985; Loizou et al., 1988). Although, as yet, there is no definitive evidence to support the causal role of anticardiolipin antibodies in fetal loss in humans, there is strong evidence from animal studies, for a possible role of anticardiolipin antibodies in the outcome of pregnancy (Blank et al., 1991). Passive transfer of anticardiolipin antibodies (mouse monoclonal or human polyclonal) to naive mice, either before breeding or during pregnancy, resulted in diminished fecundity rate, increased resorption index of embryos per pregnancy, and reduced embryo and placental weights (Blank et al., 1991). Elevated anticardiolipin antibodies have also been detected in human patients with neurological complications including focal cerebral ischemia, myelopathy, chorea, migraines, and seizures (Levine and Welch, 1987). Anticardiolipin antibodies are also present in a number of bacterial (eg. syphilis and Lyme disease) and viral (eg. Chicken Pox, mumps and AIDS) infections in humans (Vaarala et al., 1986; Matzuda et al., 1993). In Epstein-Barr Virus infected humans mainly IgM anticardiolipin antibodies are present, while in SLE patients the predominant isotype is IgG (Dostal et al., 1990). Administration of chlorpromazine to psychiatric patients has been shown to

induce anticardiolipin antibodies (Lillicrap et al., 1990).

In contrast to humans, the existence of anticardiolipin antibodies in animals used as models has not been well documented. The presence of these antibodies have been reported in SLE-prone MRL/MP/lpr/lpr (MRL/lpr) and MRL/+/+ mice (Gharavi et al., 1989) as well as (NZW x BXSB) F1 mice (Hashimoto et al., 1992). Anticardiolipin antibodies are not evident in all SLE-prone mice. For example, NZB/NZW (F1) mice, a classical murine model of SLE, do not develop these autoantibodies [13]. In this study we report that nonautoimmune strains C57BL/6J and BALB/c mice spontaneously express antibodies to cardiolipin when exposed to estrogen. We also report detectable levels of antibodies to cardiolipin in BXSB mice carrying the *yaa* gene.

Autoantibodies in immunopathologic conditions have been shown to be potentiated by estrogen (reviewed in Ansar Ahmed and Talal, 1988; Ansar Ahmed and Talal, 1990). For example, administration of estrogen to autoimmune-prone (NZB/NZW) F_1 mice markedly accelerated the disease process characterized by increased autoantibodies to DNA, severe immune complex glomerulonephritis, and earlier death compared to controls (Steinberg et al., 1979; Roubinian et al., 1987; reviewed in Ansar Ahmed and Talal, 1988). In SLE human patients, alteration of the sex hormone levels during menopause, pregnancy or while taking estrogen containing contraceptives affects the course of the disease (Oka and Vainio, 1966; Jungens et al., 1982) SLE patients also manifest abnormalities in the metabolism of sex hormones (hyperestrogenic/hypoandrogenic states) which could influence the disease process (reviewed in Lahita, 1987). Estrogen has been shown to enhance the binding of autoantibodies against nuclear autoantigens (Ro and La) to cultured human keratinocytes

(Furukawa et al., 1988). It has thus been suggested that estrogen may be an *in vivo* trigger factor for immunologic damage in cutaneous and neonatal lupus (Lahita, 1987).

While the effects of estrogen on the regulation of autoantibodies in autoimmune individuals are relatively well documented (reviewed in Ansar Ahmed and Talal, 1988; Ansar Ahmed and Talal, 1990) there is little information on the effects of this hormone on the induction or regulation of autoantibodies in normal (non-autoimmune) animals. In our previous study we reported that estrogen treatment of normal mice markedly promoted autoantibodies to bromelin-treated mouse erythrocytes (BR-ME) (Ansar Ahmed et al., 1989b). In the present study, we have investigated the role of sex hormones on the expression of IgG and IgM antibodies to cardiolipin and other phospholipids in normal miee. Together, our observations suggest that estrogen can promote the induction of autoantibodies in normal mice.

MATERIALS AND METHODS:

Mice: Male and female C57BL/6J mice were initially purchased from the Jackson laboratories (Bar Harbour, MA). They were subsequently bred in our laboratory animal facility. Female BALB/c mice were purchased from Charles Rivers Breeding Laboratories Inc. (Kensington, NY). Mice were housed in standard cages (3 to 5 mice/cage) and fed on a commercial diet devoid of sex hormones. They were maintained in 14 light/10 dark hours in temperature and humidity controlled conditions. The health of mice was routinely monitored by the veterinarian-in-charge and laboratory animals technicians. These mice remained clinically healthy throughout the study.

Sex hormonal treatment: Four to five-week old male mice were subjected to sham orchiectomy or orchiectomy procedures under pentobarbital anesthesia as described previously (Ansar Ahmed et al., 1986). These mice were given one of the following treatments, estrogen [17-β-estradiol (as prepared implants (Ansar Ahmed et al., 1986) or commercial pellets, Innovative Research of America, Toledo, OH)], 5-α-dihydrotestosterone, or empty (sham) implants (abbreviated as E2-O, DHT-O , and P-O, respectively). Estrogen and 5-α-dihydrotestosterone subcutaneous implants have been shown to release sex hormones over a period of approximately 3-4 months (Roubinian et al., 1978; Ansar Ahmed et al., 1986). Estrogen pellets were surgically placed subcutaneously. These pellets are designed to release estrogen for two months. In unrelated experiments we found no significant differences in the biological effects (eg. on seminal vesicles, bladder or thymus) of the two different types of preparations of estrogens (Ansar Ahmed, unpublished observations). Sham orchiectomized mice that were recipients of empty implants (P-S) served as internal surgical stress controls. Four-week-old prepubertal female mice were ovariectomized and treated with estrogen (E2-O♀) or empty implants (P-O♀). Non-ovariectomized females that received placebo implants served as controls (P-S♀).

Serum collection: Sera from C57BL/6, BALB/c and MRL/lpr mice were collected individually at various time periods after treatment with sex hormones and aliquots were kept at -70°C until used. Sera from BXSB mice were obtained from the laboratory of Dr. V. Dennenberg, University of Connecticut.

Antiphospholipid Antibodies: Antibodies to cardiolipin were detected by the previously reported standardized method (Gharavi et al., 1989; Harris, 1990) with minor

modifications. Briefly, each of 96-well flat bottom plates (Costar, medium-binding EIA/RIA plate) were coated with 30 μl of cardiolipin (Sigma, MO) in ethanol at a concentration of 50 μg/ml. Plates were left in the dark, at 4°C overnight to evaporate the diluent. The PBS containing 2% BSA (90 ul/well) was added for 1 hour and plates were then washed 3 times with an excess volume of PBS. Sera, derived from mice of differing sex hormone treatment, were diluted (starting at 1:100 dilution; 50 μl/well) in PBS containing 2% BSA. The samples were added to cardiolipin-coated wells in duplicate. Plates were incubated for 3 hours at room temperature. After washing with PBS, 50 μl of alkaline phosphatase conjugated Goat anti-mouse IgG (enzyme-conjugated antibodies Southern Biotechnologies Assoc.) in an optimal (1:3000) dilution in PBS - 2% PBS was added and the plates were further incubated for 1 hour at 37°C in a humid chamber. Plates were washed with PBS, developed with p-nitrophenylphosphate (Sigma, MO) in diethanolamine buffer (pH 9.8) and read after 60 minutes. In early experiments, including the studies of the expression and titers of anticardiolipin antibodies in intact male and female C57BL/6 mice (table 3.1), on the effects of male hormones on anticardiolipin antibody incidence and titer (table 3.2), and on BXSB mice (**figure 3.4**), the serum samples were diluted in PBS containing 10% adult bovine serum (ABS), and the secondary antibody used was from Caltag, (South San Francisco, CA). To avoid the potential interference of bovine β2-glycoprotein I, the sera diluent was later changed to PBS 2%BSA. In our hands, as reported, the use of ABS did not interfere with the assay. The optical density (OD) was determined at 405 nm using a kinetic microplate reader (Molecular Devices). Negative controls included wells with all reagents except serum (no serum blanks) or antigen (no-antigen blanks). Absorbencies are reported as specific OD (mean OD of sample *minus* mean OD of no serum blanks). In our experience non-specific binding of mouse sera to wells which lacked antigen (but ethanol added) was

minimal. Also "no serum" blanks were comparable with "no antigen" blanks suggesting lack of non-specific binding. In addition, eight wells of each plate always included a high titer anticardiolipin positive serum from a MRL/lpr mouse which served as a positive control. As reported earlier (Gharavi et al., 1989) we found that sera from many NZB/NZW (F1) mice were negative for anticardiolipin antibodies (but positive for anti DNA), which further validates the specificity of the assay. This stringent approach allowed us to conservatively but definitively determine the anticardiolipin levels.

In order to evaluate the influence of ABS containing β_2-glycoprotein 1 (β_2-GPl) on the binding of antibodies to cardiolipin, we diluted selected sera (5 known strong positives, 5 known borderline positives, and 5 known negatives) in PBS containing different concentrations of ABS (1,10 and 20%) or PBS-1% BSA. Appropriate controls (no serum and no antigen) for each type of buffer were included. No differences were found in the no-serum blanks that utilized buffer containing (β_2-GPI) (PBS with 1, 10 or 20 % ABS) or devoid of β_2-GPI (PBS-1% BSA). The assay was otherwise performed as before.

Inhibition studies: To access the specificity of the assay, anticardiolipin antibodies inhibition studies were performed as described earlier (Harris et al.,1985). Serum samples (1:100 dilution) from five estrogen-treated mice with high titers of anticardiolipin antibodies (1:1600 and above) were preincubated with cardiolipin, either as a solution of micelles in PBS (1 mg/ml) or bound to polystyrene plates (4.9 mg/ml in ethanol) for 1 hour at room temperature and then overnight at 4°C. Cardiolipin micelles were prepared by evaporating cardiolipin under a nitrogen stream and then resuspending them in PBS by vigorous vortexing (Harris et al.,1985). The preincubated sera were assayed for cardiolipin by ELISA as before. Appropriate controls (included in the same plate) were:

serum preincubated in PBS devoid of cardiolipin; micelles solution alone (no-serum); no-serum and no-micelles blanks; and serum that was not preincubated. The controls for the inhibition studies that utilized bound cardiolipin included sera preincubated in ethanol coated plates. Preincubation of serum with cardiolipin markedly inhibited the binding of anticardiolipin antibodies to cardiolipin (% inhibition range: 72 to 93%). Similar findings have been reported in autoimmune-prone MRL mice (Gharavi et al., 1989).

Antibodies to other phospholipids Antibodies to phospholipids [(Phosphatidylcholine, Phosphatidylinositol, Phosphatidylethanolamine or Phosphatidylserine (Sigma, MO)] were determined by the above procedures. These phospholipids, however, were dissolved in a chloroform and methanol mixture (1:3 v/v) as reported (Gharavi et al.,1989) .

Thrombocyte Count: Fresh heparinized blood from mice was added to Unopette units (Becton-Dickson). The instructions of the manufacturer were followed to enumerate the platelets in a Neubauer hematocytometer. The platelet numbers in unmanipulated mice were comparable to previous reports (Eccleston, 1977). Importantly, in order to correlate platelet numbers with antibodies to cardiolipin, paired blood and serum samples, respectively, were collected from individual mice.

Statistical Analysis: Non-parametric tests were employed given the lack of normality of the data distribution. The Mann-Whitney/ Krusckal Wallis tests were employed to test differences in absorbance between experimental groups. The Chi-Square test was used to determine the significance of the differences in the incidence of antibodies between groups. Log-transformation was employed to normalize the distribution of the titers of anticardiolipin antibodies. Spearman's Rho was used to correlate the level of

anticardiolipin antibodies and estrogen levels as well as antibody levels and platelet numbers .

RESULTS

Sex differences in anticardiolipin antibodies: Only four out of eighteen male mice had detectable levels of spontaneous anticardiolipin antibodies, compared to eleven out of nineteen female mice **(Table 3.1)**. The mean titer (natural log scale) of these autoantibodies was 1.13 in the males compared to 3.31 in females ($p = < 0.05$).

Male hormones do not augment anticardiolipin antibodies: Since male mice have lower incidence and titer of anticardiolipin antibodies compared to females, we investigated whether this is due to suppression by male sex hormones. Prepubertal male mice were orchiectomized to deplete male sex hormones and their sera were examined for anticardiolipin antibodies several months after surgery. As shown in **table 3.2**, orchiectomy had little effect on augmenting this autoantibody when compared to sham orchiectomized controls (statistically not significant. Incidence-$p=0.74$; Titers-$p=0.78$). Administration of dihydrotestosterone (DHT) implants to orchiectomized male mice had no effect on the incidence or titer of anticardiolipin antibodies. Surgical stress did not influence anticardiolipin antibodies as there was no significant difference in either the incidence or level of anticardiolipin antibodies between sham orchiectomized **(Table 3.2)** and intact mice **(Table 3.1)**.

Administration of estrogen markedly augments anticardiolipin antibodies in normal mice: In order to investigate whether the increased incidence of antibodies to cardiolipin

observed in untreated nonautoimmune C57BL/6J female mice were due to the permissive action of estrogen, prepubertal male mice were orchiectomized and given either estrogen or placebo treatment. Mice that were sham orchiectomized and received empty (placebo) implants served as controls **(Figure 3.1).** Estrogen treatment of orchiectomized mice markedly increased the level of IgG and IgM anticardiolipin antibodies. Treatment of gonadal intact male mice with estrogen also increased the expression of antibodies reactive with cardiolipin (data not shown).

The spontaneously higher expression of antibodies to cardiolipin in females is dependent upon the presence of ovaries.

To determine whether the higher incidence of IgG antibodies to cardiolipin in intact female mice was dependent upon the presence of endogenous estrogen, we assessed the expression of antibodies to cardiolipin relative to the presence or absence of ovaries (mice were ovariectomized prepubertally). The level of IgG antibodies to cardiolipin was assessed in ovariectomized and sham ovariectomized female mice that were implanted with placebos as well as in ovariectomized mice that received estrogen implants. As shown in **Figure 3.2**, the level of antibodies to cardiolipin was lower (Kruskal Wallis three way test $p < 0.005$) in ovariectomized mice receiving a placebo than in those with intact ovaries, or ovariectomized that had received estrogen implants. The level of antibodies to cardiolipin in the ovariectomized mice that were implanted with estrogen was marginally increased compared to those that had intact ovaries.

The estrogen-mediated expression of antibodies to cardiolipin in nonautoimmune mice is not restricted to C57BL/6J mice: To assess whether the induction of autoantibodies to cardiolipin in non-autoimmune mice was restricted to C57BL/6 (H-2^b) mice, we administered estrogen implants to intact female BALB/c (H-2^d) mice. BALB/c female mice treated with estrogen had significantly higher levels of antibodies of IgG and IgM isotype to cardiolipin (**Figure 3.3**).

In BXSB mice, expression of antibodies to cardiolipin is dependent on the presence of the *yaa* accelerating gene: BXSB mice is a recombinant inbred strain that develops autoantibodies and glomerulonephritis that resembles the one in human SLE. The unusual feature of this strain is that the disease is expressed earlier and more severely in the males. This is attributed to an accelerating gene in the Y chromosome, the *yaa* gene. To assess whether BXSB mice expressed antibodies to cardiolipin and whether their expression is modulated by gender, male and female BXSB mice carrying the yaa gene were tested for the expression of this antibody. Controls included male and female BXSB mice that did not carry the accelerating gene. As shown in **Figure 3.4,** the expression of anticardiolipin antibodies in these mice is regulated by the presence of the *yaa* gene and not by gender.

Estrogen also induces antibodies to other Phospholipids: We next investigated whether estrogen treatment also induces autoantibodies to other membrane phospholipids. Sera from individual mice were tested for reactivity against a number of phospholipids. As shown in **Figure 3.5,** estrogen treated mice had antibodies reactive to phosphatidylinositol, phosphatidylserine, phosphatidylcholine but not to phosphatidylethanolamine **(Figure 3.5)**. Importantly, within a given animal although

estrogen increased antiphospholipid antibodies, the extent of increase differed among phospholipids (Ansar Ahmed and Verthelyi, 1993).

Binding of anticardiolipin antibodies is not increased by ABS: ABS contains β_2-GPI [29] which has been proposed as a necessary cofactor for the binding of anticardiolipin antibodies to cardiolipin-coated polystyrene plates (McNeil et al., 1990). We investigated whether increasing concentrations of ABS in the buffer (used to dilute sera) would increase the binding of the anticardiolipin antibodies. This was not the case as 14 out of 15 sera had lower binding (Mean reduction in the ODs at 1:100 dilution: 47%) when diluted in PBS containing 10 or 20% ABS compared to PBS containing 1% ABS or 1% BSA. Sera incubated in PBS 1% ABS had binding of anticardiolipin antibodies comparable to PBS-1% BSA. Importantly none of the sera that were negative for anticardiolipin antibodies when incubated in PBS-1% BSA or PBS-1% ABS became positive when the concentration of ABS in the buffer was increased.

Anticardiolipin antibody levels and Platelet counts: There was no correlation between the levels of anti-cardiolipin antibodies and platelet numbers in mice ($r=0.033$). This lack of correlation was observed regardless of the hormonal treatment (eg. $r=0.005$ for estrogen treated mice).

DISCUSSION

In recent years there has been a surge of interest in antiphospholipid antibodies in general and anticardiolipin antibodies in particular (reviewed in Parke, 1989; Macworth-Young, 1990; Harris, 1990). Anticardiolipin antibodies have been detected in humans with

diverse clinical conditions particularly autoimmune diseases (eg. SLE, APS). Thus far, anticardiolipin antibodies have been detected only in certain autoimmune-prone, MRL/lpr and MRL+/+ (Gharavi, 1989) and (NZW X BXSB)F1 (Hashimoto et al., 1992). We report IgG anticardiolipin antibodies in BXSB strain of mice, which develops lupus-like disease with lymphadenopathy. Normal strains of mice such as BALB/c have been reported to be negative for anticardiolipin antibodies (Gharavi et al., 1989), even though they produce autoantibodies of other specificities (Dighiero et al., 1983; Underwood et al., 1985).

In this study, we find the presence of low levels of antibodies that react to cardiolipin in unmanipulated non-autoimmune C57BL/6J mice, particularly in females (**Table 3.1**). Male hormones appear to have minimal effect in the regulation of these autoantibodies since neither prepubertal orchiectomy nor administration of dihydrotestosterone to orchiectomized mice significantly altered the incidence or levels of anticardiolipin antibodies (**Table 3.2**). In contrast, administration of estrogen to orchiectomized mice profoundly increased the incidence and titers of this antibody. Further, estrogen markedly increases the incidence and levels of anticardiolipin antibodies in both gonadal intact female and male mice (Figs. 1 & 2). These antibodies were found to be consistently increased in estrogen-treated mice when examined at different ages. It must be emphasized that other than hormonal treatment these mice were not injected with cardiolipin or any autoantigens or preparations from infectious agents. Mice had increased anticardiolipin antibodies at a time when estrogen had returned to normal levels (data not shown). It thus appears that initial, rather than sustained, exposure to estrogen is sufficient to induce anticardiolipin antibodies. Long-term sequential studies involving short exposure of non-autoimmune mice to estrogen are underway to confirm this aspect.

Estrogen treated non-autoimmune mice also developed antibodies which bound to other membrane phospholipids. Overall, antibodies to phosphatidylinositol and phosphatidylserine tended to be moderate to high, while those to phosphatidylethanolamine were barely detectable. Inhibition experiments performed by preincubating sera with cardiolipin or phosphatidylserine (plate-bound or in micelle solution) showed that the antibodies to cardiolipin crossreact with those to phosphatidylserine and phosphatidylinositol, but not with zwitterionic phospholipids (Ansar Ahmed et al., 1993). However, we observed that some mice have high levels of antibodies to cardiolipin but not phosphatidylserine or phosphatidylinositol and *vice versa*. These results suggest that there could well be two types of antibodies: (1) low titer antibodies which may be polyreactive (reactive to other phospholipids), and (2) high titer antibodies which may be monoreactive.

The contribution of the genetic background of C57BL/6J and BALB/c to the development of low levels of anticardiolipin antibodies is not known, however, these mice are regularly considered as nonautoimmune. However, under the influence of estrogen, the expression of these antibodies are markedly augmented. Several mechanisms may explain these observations: 1) estrogen may act directly or indirectly on B cells to promote clonal expansion of autoreactive B cells producing antibodies to phospholipids. The indirect sites (eg. hypothalamus-hypophysis, macrophages) through which sex hormones mediate their effects on the immune system have been reviewed in detail elsewhere (Dighiero et al., 1983; Grossman, 1984, Ansar Ahmed and Talal, 1988); 2) estrogen may affect T cells to alter the production of cytokines which act on B cells (eg. IL-2, IL-4, IL-5, IL-6) to promote autoantibody production; 3) estrogen may be toxic to cells thereby exposing phospholipids such as cardiolipin to provoke an immune

response. The above hypotheses are currently under investigation in our laboratory. Finally, the possibility that estrogen may activate an unknown latent virus or other infectious agents which could promote autoimmunity cannot be ruled out.

Beta glycoprotein-I (β_2-GPI), a known inhibitor of the intrinsic coagulation pathway and prothrombinase activity of activated platelets, has been shown to be present in ABS (Matsuura et al., 1991. A recent study reported that affinity purified anticardiolipin antibodies bind to cardiolipin on polystyrene plates only in the presence of β2-GPI (McNeil et al., 1990). Subsequent studies show that the degree of enhancement of the binding of anticardiolipin to cardiolipin in the presence of β_2-GPI is variable in SLE sera (Gharavi.). Further, β2-GPI was shown to block the binding of these antibodies in syphilis patients (Pierangeli et al., 1992). Our results show that: (i)- increasing concentrations of ABS in the buffer do not increase the binding of anticardiolipin antibodies. Rather, it decreased the binding suggesting that ABS is acting as a blocking agent. (ii)- the use of diluting buffer devoid of β2-GPI does not diminish the incidence of these antibodies. Although it is possible that estrogen may also increase β2-GPI in the sera of mice, the above data suggests that β2-GPI does not increase the binding of estrogen-induced anticardiolipin antibodies. Moreover, throughout the study all sera were incubated with the same concentration of ABS in the diluting buffer. Further studies utilizing β_2-GPI are planned to test this aspect.

We found, as observed in MRL/lpr mice (Gharavi et al., 1989), that the levels of anticardiolipin antibodies did not correlate with platelet numbers in C57BL/6J mice despite the association of these antibodies with thrombocytopenia in humans.

In a brief report on human studies, it was observed that a small group of women with vascular complications who used estrogen containing oral contraceptives did not develop anticardiolipin antibodies (Bruneau et al., 1986). This apparent discrepancy with the present findings may be due to obvious differences in the species, disease/health status of the subjects, or dose, purity and route of administration of estrogen between the two studies. Further large studies are needed to definitively determine whether or not estrogen promotes antiphospholipid antibodies in humans. It would also be interesting to determine whether post-menopausal patients on estrogen therapy develop anticardiolipin antibodies. Our present studies are in agreement with previous observations that estrogen induces B cells that produce anti-erythrocyte (bromelin pretreated) autoantibodies in normal mice (Ansar Ahmed et al., 1989). Further, this study also supports a vast body of literature which documents that estrogen treatment of normal rodents augments levels of total immunoglobulins and specific antibodies to many exogenously administered foreign antigens (Butterworth et al., 1967; Dighiero et al.,1983; Brick et al., 1985; Myers and Petersen, 1985; Underwood et al., 1985). Moreover, estrogen-treatment of autoimmune mice has been shown to promote antibodies to a variety of autoantigens (Ansar Ahmed and Talal, 1988; Roubinian et al., 1978; Steinberg et al., 1979).

It is important to note that estrogen can potentiate normal B cells to spontaneously produce autoantibodies. Further, this data supports the concept that estrogen may alter normal immunoregulatory pathways to induce B cell hyperactivity. The pathogenic significance (eg. thrombosis or fetal loss) of anticardiolipin antibodies induced by estrogen needs to be determined.

Acknowledgements

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Table 3.1: Normal male mice have decreased incidence and levels of anticardiolipin antibodies compared to females

GROUP	n	Incidence (%)	Mean titer ± SEM	Median titer
MALE	18	22.2	1.13 ± 0.52	0
FEMALE	19	57.8	3.31 ± 0.64	4.6

Statistical Significance: Incidence $p = <0.05$ Titers $p = <0.05$

Serum of normal male and female mice was used to determine anticardiolipin antibodies by ELISA. (The age of mice is as follows: Females: Mean-10.4 mo.; Median-8.8 mo; range-3 to 22 mo. Males: Mean-10.8 mo.; median-9.8 mo.; range-3 to 22 mo.)

Table 3.2: Depletion of male hormones do not induce the expression of IgG antibodies to cardiolipin

GROUP	n	Incidence (%)	Mean titer ± SEM	Median titer
P-S	30	30	1.68 ± 0.85	0
P-O	28	20	1.17 ± 0.78	0
DHT-O	21	33.3	1.78 ± 0.8	0

Statistical Significance: Incidence p = NS Titers p = NS

Sera from orchiectomized mice that were treated with 5α-dehydrotestosterone (DHT) or placebo implants were assayed for anticardiolipin antibodies by ELISA. Mice that were sham orchiectomized and were treated with placebo implants served as controls. Mice were matched relative to age (Mean-7.8 mo.; median-9 mo.; range-3 to 11 mo.)

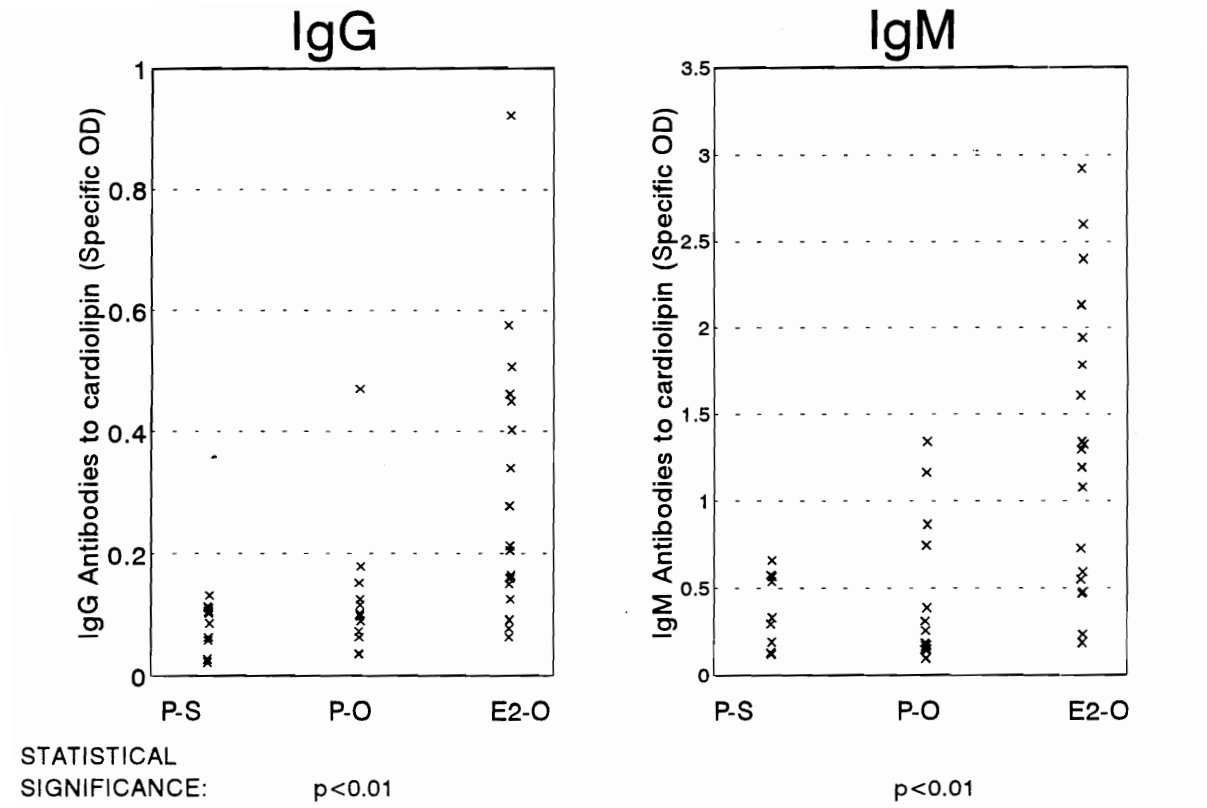
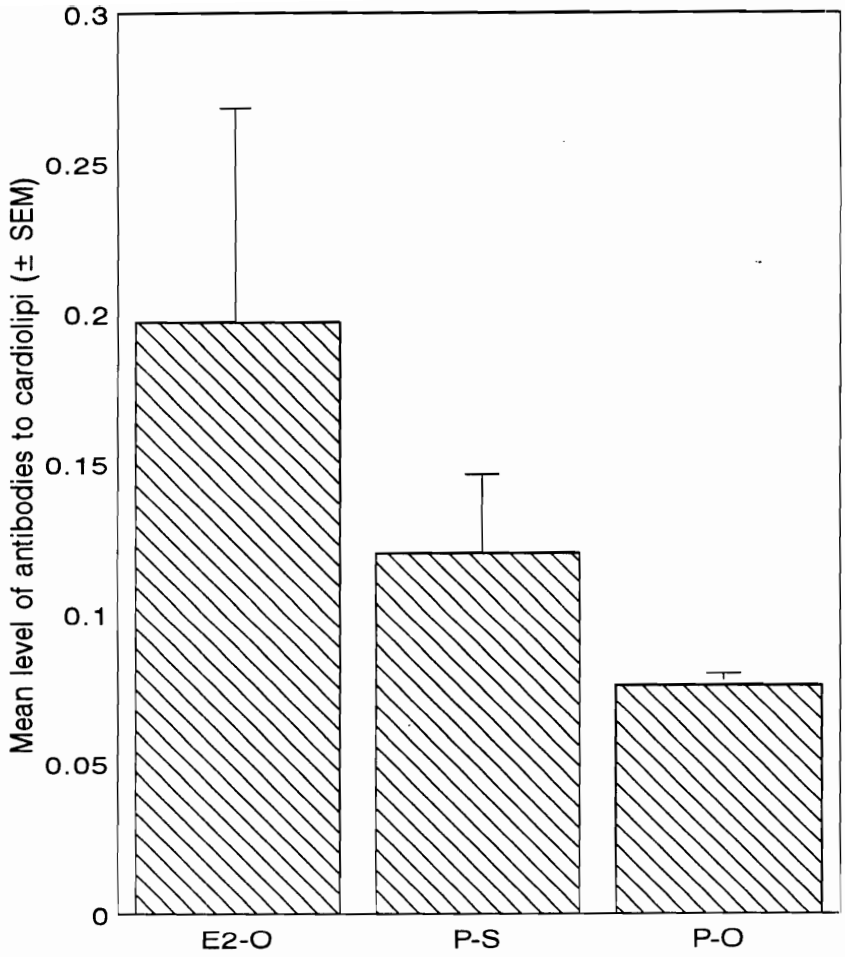


Figure 3.1: Normal male C57BL/6J mice were prepubertally orchiectomized (O) or sham orchiectomized (S) at 4-5 weeks of age and given empty (P), or estrogen (E_2) implants. E2-O mice had significantly higher levels of IgG and IgM anticardiolipin antibodies ($p < 0.01$).



Statistical significance:
Three way Kruskal Wallis: $p<0.005$
Dunn'multiple comparisons: E2-O vs P-O $p<0.01$;
E2-O vs P-S $p>0.05$

Figure 3.2: Normal female prepubertal mice were ovariectomized and treated with empty (sham) or estrogen implants. Non-ovariectomized female mice that received placebo implants served as controls. Sera from mice were tested for anticardiolipin antibodies.

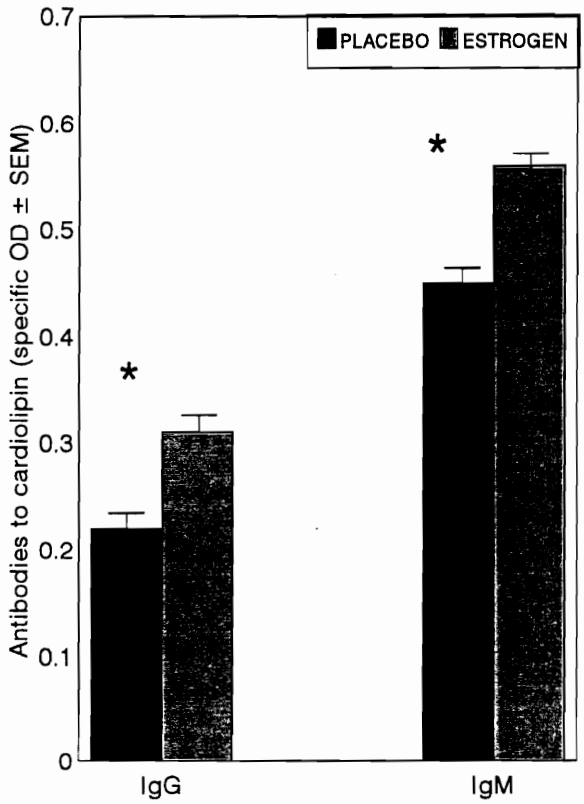
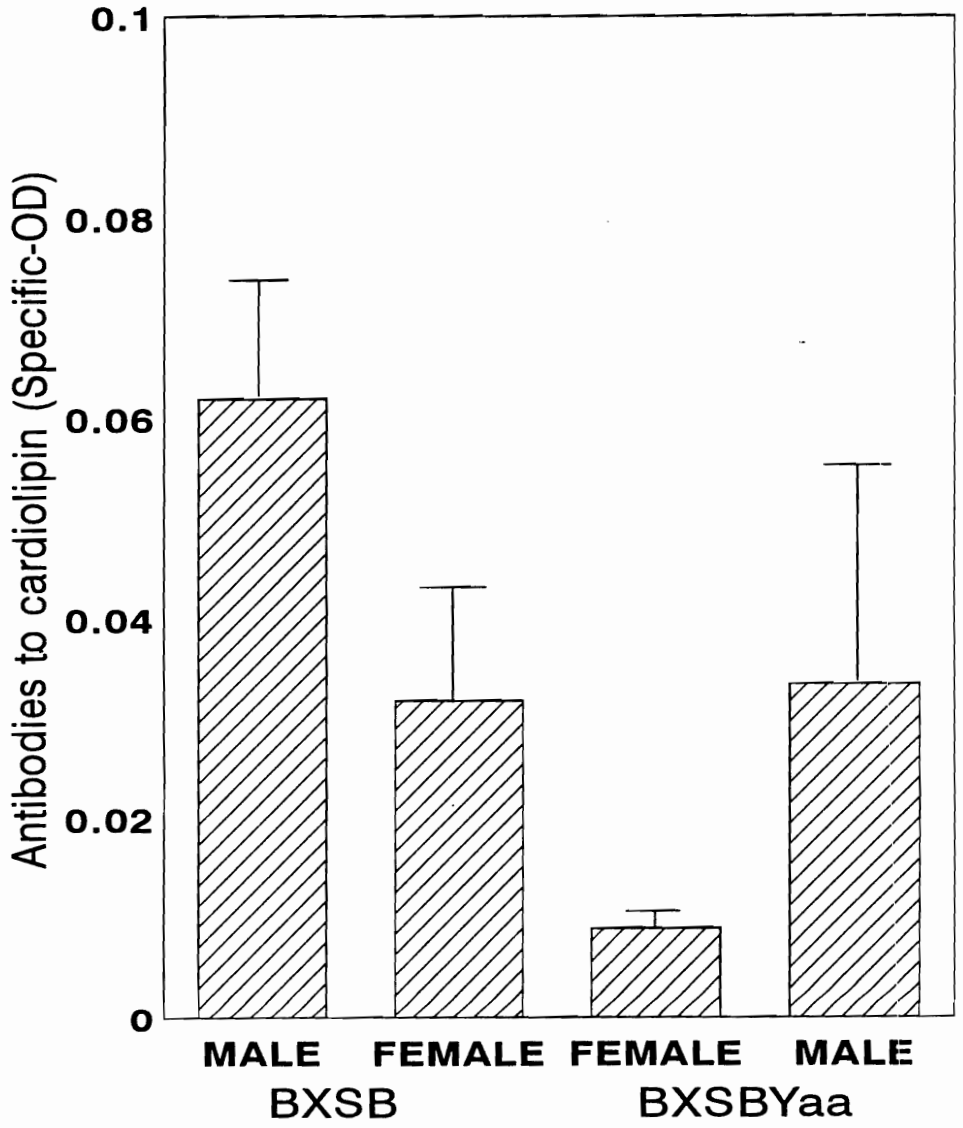


Figure 3.3: Sera from BALB/c female mice that were treated prepubertally with either estrogen (n=20) or empty (n=20) implants for 30 days were tested for the expression of antibodies to cardiolipin. Mice treated with estrogen had significantly higher levels of IgG and IgM antibodies to cardiolipin ($p < 0.01$).



STATISTICAL SIGNIFICANCE:

BXSB MALE X FEMALE P = .0019
MALE BXSB X BXSBYaa P = .009
FEMALE BXSB X BXSBYaa P > .10
BXSBYaa MALE X FEMALE P > .10

Figure 3.4: The expression of antibodies to cardiolipin was assessed in autoimmune-prone female and male BXSB mice. Mice were grouped according to the expression of the yaa gene.

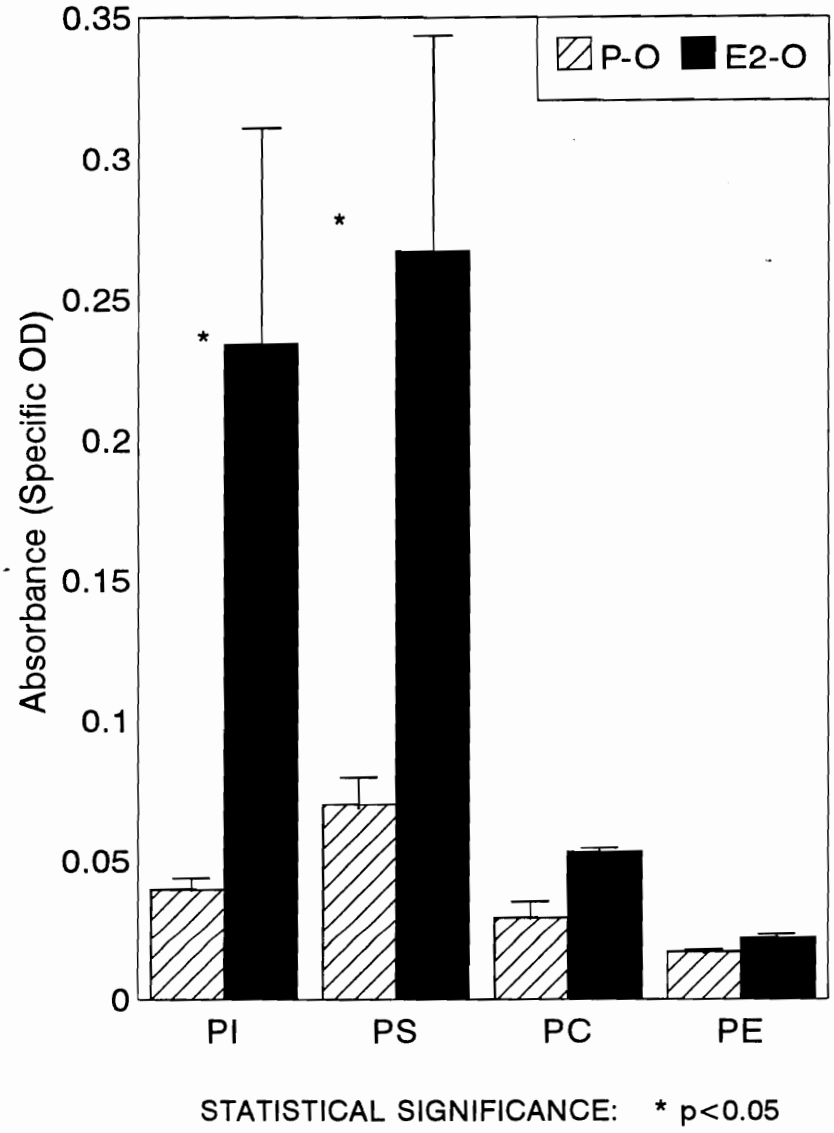


Figure 3.5:

Sera from orchiectomized male C57BL/6 mice treated with estrogen (n=12) or placebos (n=8) were tested for the expression of antibodies to membrane phospholipids phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

CHAPTER 4

17β-ESTRADIOL BUT NOT 5α-DIHYDROTESTOSTERONE AUGMENT ANTIBODIES TO dsDNA IN NON-AUTOIMMUNE C57BL/6J MICE

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ABSTRACT

The influence of sex hormones on the immune response to foreign as well as self antigens is now recognized. In this study, we investigated the influence of gender and sex hormones on the development of antibodies to dsDNA in non-autoimmune C57BL/6J mice. IgG anti-dsDNA antibodies are commonly present in lupus patients and several autoimmune-prone murine strains. We found that C57BL/6J mice have detectable antibodies (IgM and IgG but not IgA) to dsDNA. Interestingly, the incidence and level of IgG anti-dsDNA antibodies were lower in male than in female mice. Orchiectomy or administration of 5α-dihydrotestosterone to orchiectomized male mice had minimal effects on these antibodies. In contrast, administration of 17β-estradiol to orchiectomized or intact males significantly increased both incidence and levels of anti-dsDNA antibodies. In female mice, ovariectomy decreased while administration of estrogen augmented the incidence and levels of anti-dsDNA antibodies in ovariectomized as well as in intact female mice. Kinetic studies revealed that estrogen treatment of male and female mice induced earlier and sustained expression of IgG anti-dsDNA antibodies. IgG subisotype analysis showed IgG2b to be predominant. In summary, together our findings suggest that estrogen but not dihydrotestosterone promote anti-dsDNA antibodies in normal mice.

INTRODUCTION:

Accumulating evidence shows that there is a close interaction between the immune and endocrine systems (Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990; Grossman, 1985). In humans, women in general have higher levels of immunoglobulins, and mount stronger responses to a variety of heteroantigens than men (reviewed in Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990; Grossman, 1985). This allows them to respond better to microbial and non-microbial antigens compared to their male counterparts (reviewed in Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990; Grossman, 1985). The greater immune responsiveness in women is also evident in their higher susceptibility to autoimmune diseases, especially during their reproductive years. For example, women to men susceptibility ratios for Systemic lupus erythematosus (SLE), Sjogren syndrome (SS), and rheumatoid arthritis are 10:1, 9:1 and 2-7:1, respectively (Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1993). In autoimmune patients, the course of the disease is modulated by fluctuations in hormonal status such as pregnancy, menses or the use of oral contraceptives (Ansar Ahmed et al., 1985; Schuurs and Verheul, 1990; Ansar Ahmed and Talal, 1993; Hazes et al., 1990b). Further, female SLE patients have an abnormal metabolism of their sex hormones resulting in an increased production of 16 α-hydroxyesterone and estrol metabolites which are thought to induce a chronic hyperestrogenic state. Male SLE patients have reduced levels of testosterone and dehydroepiandrosterone (DHEA) and DHEA-sulfate (Ansar Ahmed and Talal, 1993; Lahita, 1990). The above observations suggest that sex hormones may be involved in the pathogenesis of autoimmune diseases.

Analogous to the human situation, gender differences in immune capabilities have also

been noted in a number of experimental laboratory animals (reviewed in Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990; Grossman, 1985; Ansar Ahmed and Talal, 1990). For example, female mice have higher immunoglobulin levels and increased antibody responses to a variety of antigens compared to males. Further, female laboratory animals have a reduced incidence of tumors and reject allografts more rapidly than males (Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1993). This suggests a stronger humoral and cell-mediated immunity compared to males. The influence of sex steroids has been observed in several experimental animal models for autoimmune diseases [organ and non-organ specific, (Reviewed in Ansar Ahmed et al., 1985a; Ansar Ahmed and Talal, 1993)]. Female NZB x NZW F_1 (B/W) mice, a classical model for SLE, develop autoantibodies, lymphadenopathy, arthritis, immune complex glomerulonephritis and die earlier than their male counterparts (Siiteri et al.,1980). Administration of estrogen to B/W males resulted in accelerated development of severe nephritis accompanied by higher levels of anti-DNA antibodies and enhanced mortality (Roubinian et al, 1978). In contrast, dihydrotestosterone (DHT) treated mice had reduced levels of anti-DNA antibodies and their survival was prolonged. In other murine models of SLE, including NZB x SJL/J, NZB x CBA, NZB x C3H and NZB x DBA/2, androgens have been shown to retard, while estrogens accelerate their expression of autoimmune disease and mortality (Ansar Ahmed and Talal, 1993). Sex hormone regulation of autoimmune reactivity has also been noticed in other models of autoimmune disease, including those prone to Insulin Dependent Diabetes Mellitus (NOD mice; Yagi et al.,1991), polyarthritis (LEW/N rats) or autoimmune thyroiditis (PVG/c rats; reviewed in Ansar Ahmed and Talal, 1991). The effects of estrogen on the regulation of autoantibodies in autoimmune individuals is relatively well documented (Ansar Ahmed et al., 1985a; Shuurs and Verheul, 1990; Ansar Ahmed and Talal, 1993; Ansar Ahmed

and Talal, 1990). However, there is little information on the effects of this hormone on the induction or regulation of autoantibodies in healthy individuals. Therefore, in this study, we investigated for the presence of antibodies to dsDNA in C57BL/6J mice. This strain is currently considered as non-autoimmune and is employed as a control in numerous immunologic studies. However, C57BL/6J mice have been shown to develop autoimmune type lesions in old age (Hayashi et al., 1989; Ansar Ahmed, 1994). Also, this strain constitutes part of the genetic background of mice that spontaneously develop autoimmune disease (BXSB/Mp Yaa mice; Ansar Ahmed, 1994). Since autoimmune diseases are multifactorial (genetic, environmental, hormonal, infectious factors, etc.), C57BL/6J mice may represent a good model to study the influence of sex steroids on healthy individuals who may have some predisposing factors for autoimmune disease in their background.

In previous studies we reported that estrogen treatment of normal mice markedly promoted autoantibodies to bromelin treated erythrocytes (Ansar Ahmed et al., 1989b), cardiolipin and other phospholipids (Ansar Ahmed and Verthelyi, 1993; Ansar Ahmed et al., 1993). In the present study, we have investigated the influence of sex hormones on the expression of IgG antibodies to dsDNA. Antibodies to DNA are present in normal healthy individuals as a part of their natural autoantibody repertoire. These antibodies mainly belong to IgM class, have low affinity, react with several antigens, and have undergone little somatic mutation (Hahn and Tsao, 1993). Antibodies to dsDNA are commonly present in SLE and SS. These antibodies have undergone a high number of antigen-driven somatic mutations, react primarily with dsDNA, and are mainly of IgG1 isotype in humans, or IgG2a, IgG2b or IgG3 isotype in mice (Hahn and Tsao, 1993; Gilbert et al., 1992; Takahashi et al., 1991). Our observations suggest that estrogen can

promote the induction of IgG anti-dsDNA antibodies in otherwise healthy mice.

MATERIALS AND METHODS:

Mice: Male and female C57BL/6J mice were initially purchased from Jackson Laboratories and subsequently bred in our laboratory animal facility.. Mice were fed on a commercial diet devoid of sex hormones, and housed in standard cages (3 to 5 mice/cage) with a 14 light/10 dark hours schedule. The health of the mice was routinely monitored by the veterinarian-in-charge and laboratory animal resources technicians.

Sex hormonal treatment: Four to five-week old mice were given one of the following treatments under pentobarbital anesthesia as described in **table 4.1**. Mice were gonadectomized (orchiectomized or ovariectomized) as described previously (Penhale and Ansar Ahmed, 1981). Silastic implants (Dow Corning Co., MI) or commercial pellets (Innovative Research of America, Toledo, OH) containing 17β-estradiol (E2), or 5α-dihydrotestosterone (DHT) were surgically placed subcutaneously as reported earlier (Ansar Ahmed et al.,1989b; Ansar Ahmed et al, 1986). Mice with and without gonadectomies that received empty [Placebo (P)] implants served as controls. The commercial pellets are time-release capsules designed to slowly release sex hormones for 2-3 months. Silastic implants [(7mm long, containing 6-8 mg of 17β-estradiol (Sigma, MO)] also release hormones slowly for several months (Roubinian et al, 1978; Ansar Ahmed et al, 1986). In previous studies we found no significant difference between the two types of estrogen preparations in terms of induction of anticardiolipin antibodies (Ansar Ahmed et al.,1989b) or T cell proliferative response (Ansar Ahmed, Gogal and

Walsh, unpublished observations). In selected experiments, gonadal intact mice received estrogen or placebo implants (table 4.1). In mice utilized for sequential studies implants (estrogen or placebo) were surgically removed (under pentobarbital anesthesia) after 60 days to ensure that there was no residual release of 17β-estradiol.

Serum collection: Serum from each mouse was collected retroorbitally and kept frozen at -70°C until tested for anti-dsDNA antibodies. To assess estradiol levels, because of small volume of the serum obtained, it was necessary to pool sera from mice of each hormonal treatment group (3-6 mice per group). The samples were analyzed by radioimmunoassay by Dr T.J. Reimer (Diagnostic Laboratory, New York College of Veterinary Medicine, NY). Frozen sera from (NZB x NZW)F_1 and BXSB/MP mice were obtained from the laboratory of Dr. V. Dennenberg, University of Connecticut.

Antibodies to DNA: Antibodies to dsDNA were detected according to previously reported standardized methods (Gharavi et al, 1989; R.L.Rubin, Scripps Clinic and Research Foundation, California, personal communication) with minor modifications. In our initial studies Poly-L-Lysine (Sigma, St.Louis, MO; 50 ul/ml) was utilized to coat 96-well flat bottom plates (High-binding EIA/RIA plate, Costar, Cambridge, MA). Plates were incubated for 1 hour at room temperature and then coated with 70 μg/ml of calf thymus dsDNA (Sigma, MO) overnight at 4°C. To remove residual ssDNA, plates were incubated for 1 hour at 30°C in a humid chamber with S1 Nuclease (50 U/ml in S1 Nuclease Buffer). After washing with PBS, the wells were blocked with PBS containing 2% bovine serum albumin (BSA; 75 ul/well) for 90 minutes. Mice sera, at 1:100 dilution (50 ul/well) in PBS containing 10% heat-inactivated adult bovine serum (ABS, JRH Bioscience, KS) were then added to the wells. Plates were incubated for 3

hours at room temperature. After washing, 50 ul of alkaline phosphatase conjugated goat anti-mouse immunoglobulin (heavy chain specific, Caltag, South San Francisco, CA), in an optimal (1:1500 or 1:3000) dilution in PBS - 10% ABS was added and the plates were further incubated for 1 hour at 37°C in a humid chamber. Plates were washed again, developed with p-nitrophenylphosphate (Sigma, MO) in diethanolamine buffer (pH 9.8) and read after 60 minutes (405 nm, Molecular Devices, Menlo Park, CA). Negative controls included wells with all reagents except serum (no serum blanks), with known negative sera or with no antigen (no antigen blanks). Since known negative sera had similar optical densities (OD) as no serum blanks, no serum blanks were utilized to control for background in most instances. In addition, eight wells of each plate containing known positive sera from BXSB or MRL/lpr mice served as the positive control. Murine antibodies have recently been shown to bind non-specifically to poly L-lysine coated wells without DNA (Kroubouzos et al., 1992). The coating method was changed to 2 μg/ml methylated BSA in PBS to decrease the possibility of non-specific binding. The ODs obtained utilizing Poly-L-lysine were slightly higher than the ones obtained utilizing methylated BSA (for the same sera); the differences between treatment groups, however, were consistent. To assess the specificity of the assay, inhibition studies were performed utilizing monoclonal antibodies to dsDNA (s83) and to ssDNA (s10) that were kindly provided by Dr T. Marion, Univ. of Tennessee. Supernatants from these cell lines were preincubated on plates coated with increasing concentrations of calf thymus dsDNA (0 to 700 ug/ml). After incubating for 2 hours at room temperature, the supernatants were aspirated and tested for the presence of antibodies to dsDNA. Preincubation with increasing concentrations of dsDNA absorbed out up to 90% of the anti-dsDNA antibodies binding to dsDNA coated plates (**Figure 4.1**).

Analysis of immunoglobulin isotypes and subisotypes: Serum immunoglobulin levels were estimated by ELISA. The procedure followed was similar to the one described above with the following differences: (i) Plates were coated with 1 ug/ml of heavy chain specific goat-anti-mouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b or IgG3 antibodies (Caltag,CA), in bicarbonate buffer; (ii) plates were blocked with PBS containing 1% BSA; and (iii) the conjugated antibodies utilized were alkaline phosphatase conjugated goat anti-mouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3, respectively (Caltag, CA).

Expression of ELISA data: Two methods of data analysis were chosen for this study: (i) incidence and (ii) Specific OD. Incidence is a *qualitative* assessment of anti-dsDNA antibodies - (ie, presence or absence). The cutoff point was determined as 3 SD above the mean OD of eight background control wells included on each plate. Specific OD (OD of the sample *minus* mean OD of the background controls) provide a *semi-quantitative* estimation of the antibody concentration.

Statistical Analysis: Non-parametric tests were employed given the lack of normality of the distribution of the data. Accordingly, the data is described as median values of the distribution (therefore standard errors are not indicated). Chi-Square test was used to determine the differences in incidence of antibodies between groups. The Mann-Whitney test was employed to test differences in the seric level of antibodies between groups.

RESULTS

Normal C57BL/6J have low levels of IgG antibodies to DNA: To assess whether non-autoimmune C57BL/6J mice expressed these antibodies, sera of sixteen female and eighteen male mice were tested by ELISA for the presence of IgG antibodies to dsDNA. Two of eighteen males (11.11%) compared to ten out of sixteen females (62.5%) showed detectable levels of these antibodies ($p < 0.01$). As shown in **figure 4.2a,** the level of anti-dsDNA antibodies was also significantly higher for the female C57BL/6 mice than their male counterparts (0.008 specific OD and 0.006 specific OD respectively, $p < 0.05$). The levels of antibodies to dsDNA in autoimmune-prone mice have been included in **figure 4.2b** for reference. Previous work had shown that autoimmune disease-prone (NZB x NZW)F_1 and BXSB/MP mice [carrying the Yaa (Y chromosome-linked accelerating) gene] manifest IgG antibodies to dsDNA (Hahn, 1993).

Male hormones have minimal effects on the expression of IgG antibodies to dsDNA: The lower production of IgG antibodies to dsDNA in male C57BL/6J mice was not due to a protective effect of male hormones, since orchiectomized mice that received a placebo implant (P-O♂) did not differ from the placebo-treated sham orchiectomized (P-S♂) group (0.008 specific OD and 0.007 specific OD, respectively; $p > 0.10$; **Figure 4.3).** Further, treatment of orchiectomized mice with DHT did not alter the level (0.011 specific OD; $p > 0.10$) of anti-dsDNA antibodies. There was no statistical difference

between the level of anti-dsDNA antibodies of the surgical controls (P-S♂ = 0.007 specific OD; **figure 4.3)** and that of the untreated males (0.006 specific OD; **figure 4.2a**; $p < 0.10$).

In male C57BL/6J mice, estrogen induces IgG antibodies to dsDNA: Administration of estrogen to male mice, with or without testis, had a significant enhancing effect on these autoantibodies **(Figure 4.3).** The median level of antibodies to dsDNA for mice in the E2-O♂ treated group (0.021 specific OD) was significantly higher than that of their controls (P-O♂ = 0.008 specific OD; $p < 0.005$). Mice that received estrogen but were not orchiectomized (E2-♂) had levels of antibodies to dsDNA (0.029 specific OD) similar to those observed in the E2-O♂ treated group ($p > 0.10$; data not shown). The mean level of 17β-estradiol for untreated male mice was 59.3 pg/ml, for P-O♂ was 56.6, pg/ml while that of E2-O♂ was 211 pg/ml. This value was comparable to that of E2-O♀ (193.7 pg/ml).

In female C57BL/6J mice, the expression of antibodies to dsDNA is markedly influenced by estrogen: Since estrogen promoted the expression of IgG anti-dsDNA antibodies in male mice (E2-O♂), we next determined whether estrogen would further increase the production of these autoantibodies in female mice. Twenty three female mice received estrogen implants. As observed in males, administration of exogenous estrogen to intact female mice (E2-♀ = 0.015 specific OD) doubled the level of these antibodies

when compared to female mice that received placebo implants (P-♀ = 0.007 specific OD; $p<0.01$; **Figure 4.4a)**.

The effect of estrogen in the absence of ovaries was then determined. Female mice were ovariectomized and given either a 17β-estradiol (E2-O♀ n=10) or a placebo implant (P-O♀ n=10). An additional group of 10 female mice that were sham ovariectomized and received a placebo (P-S♀) served as surgical controls. The group of mice that was ovariectomized (P-O♀) and presumably had reduced levels of female hormones, had lower levels of IgG antibodies to dsDNA (0.004 specific OD). There was no statistical difference in anti-dsDNA antibodies between P-O♀ and their surgical controls (P-S♀ = 0.005 specific OD; $p>0.10$; **Figure 4.4b**). All ovariectomized female mice that received estrogen implants, however, had significantly higher levels of antibodies to dsDNA (0.026 specific OD $p<0.0001$). Together, these studies strongly suggest that estrogen promotes these autoantibodies.

Estrogen accelerates the expression of antibodies to dsDNA: Sequential studies were performed to study the kinetics of expression of these antibodies relative to age and estrogen treatment. Prepubertal (4-5 weeks) female (E2-♀, 13 mice *per* group) and orchiectomized male (E2-O♂, 9 mice *per* group) mice received 17β-estradiol implants. An equal number of mice received placebo implants (P-♀ and P-O♂). All implants were surgically removed 9 weeks post-implantation to ensure mice would not be exposed to

more exogenous estrogen. Antibodies to dsDNA (IgG, IgM and IgA) were measured at the time of administration of the implant (4-5 weeks of age), upon removal of the implant (13-14 weeks of age), and at weeks 23 and 32. None of the mice had either IgM, IgG or IgA anti-dsDNA antibodies at the time of administration of the implant. At the time of removal of the implant over 80% of the estrogen-treated mice had detectable levels of IgM antibodies, compared to less than 50 % of the mice that received placebo implants. Estrogen treatment of mice also induced earlier and sustained expression of IgG anti-dsDNA antibodies in both male and female mice **(Figure 4.5)**. The incidence of anti-dsDNA antibodies in the female mice that received estrogen implants increased sharply with the administration of the hormone and remained elevated throughout the study. Interestingly, the incidence of antibodies to dsDNA in female mice that received a placebo increased over time and by week 53 was similar to that of estrogen-treated mice (data not shown). In orchiectomized males that received a placebo, the incidence remained below 35% compared to over 55% of those that received estrogen implants. Overall, estrogen-treated mice attained earlier, higher and more sustained levels of anti-dsDNA antibodies (mainly IgG) than the controls. IgA anti-dsDNA antibodies were not detectable in this strain with the exception of one estrogen-treated female at week 23 (data not shown).

Analysis of isotype and subisotype of anti-dsDNA antibodies in estrogen-treated mice: C57BL/6J mice had IgM and IgG antibodies reactive to dsDNA. IgA antibodies

were not detectable. It is not possible to directly compare the level of the different isotypes and subisotypes of anti-dsDNA specific antibodies because various reagents with differing degrees of sensitivity were employed. Nevertheless, a comparison between groups, estrogen-treated and Placebo-treated mice, could be made. Upon estrogen treatment, female mice significantly increased the level of anti-dsDNA antibodies of IgM and IgG isotypes ($p < 0.001$ and $p < 0.005$ respectively; **figure 4.6**). Although antibodies to dsDNA of all four subisotypes of IgG were augmented by treatment of female mice with estrogen, the largest increase corresponded to IgG2b ($p < 0.001$). In males, estrogen treatment also induced an increase in IgG anti-DNA antibodies ($p < 0.005$). IgG subisotype characterization indicated that estrogen increased anti-dsDNA antibodies of IgG2b and to a lesser extent IgG1 and IgG2a subisotypes (**Figure 4.6**).

DISCUSSION

Anti-DNA antibodies (mainly IgM) are present in normal individuals as part of their natural antibody repertoire. Antibodies to dsDNA (IgG) are commonly present in SLE patients. IgG anti-dsDNA antibodies have been found to correlate with the progression of the disease, and there is evidence of their association with tissue damage (Hahn and Tsao, 1993; Emlen et al., 1986). Their precise pathogenic role, however, has remained elusive (Hahn and Tsao, 1993; Emlen et al., 1986). DNA is a poor antigen and attempts to induce these antibodies by direct inoculation of native DNA into animals have been

largely unsuccessful. Recently, however, diverse approaches have been utilized to induce or enhance the production of anti-DNA antibodies in normal mice including inoculation with: heterologous (bacterial) DNA (Glikeson et al., 1991), DNA bound to immunogenic peptides (e.g. Fus-1; Desai et al., 1993), or inoculation with pathogenic anti-idiotype (16/6) antibody (Blank et al., 1990). Despite the low immunogenicity of DNA, the characteristics of anti-DNA antibodies in SLE appear to be the result of antigen-driven, clonally selected, specific B cell stimulation (Desai et al.,1993).

In this study, we find that unmanipulated non-autoimmune C57BL/6J mice, particularly females (figure 4.2a), have low levels of antibodies that react to dsDNA. Male hormones appear to have minimal effects on the regulation of these autoantibodies since neither prepubertal orchiectomy nor administration of 5α-dihydrotestosterone to orchiectomized mice significantly altered the level of these antibodies (Figure 4.3). In contrast, administration of estrogen augmented the level of anti-dsDNA antibodies. Further, depletion of endogenous estrogen by ovariectomy in female mice resulted in lower anti-dsDNA antibody levels. Thus, the above studies suggest that estrogen promotes anti-dsDNA antibodies in non-autoimmune C57BL/6J mice, while male hormones do not. The autoantibody nature of the anti-dsDNA antibodies from estrogen-treated mice was assessed by comparing their binding to dsDNA from calf thymus (routinely utilized for testing anti-dsDNA antibodies), with dsDNA from liver of estrogen-treated mice and placebo-treated mice dsDNA (data not shown). The results

show very similar binding patterns indicating the autoantibody nature of these antibodies. Studies are underway to assess whether these antibodies have a pathologic role.

Sequential studies showed that young animals exposed to estrogen produce IgM and especially IgG anti-dsDNA autoantibodies earlier and in higher amounts. Interestingly, the incidence of anti-dsDNA antibodies in E2-O♂ increased after the implants were removed. This suggests that exposure to estrogen for a short period (9 weeks) has a long-lasting effect on the immune system. Further, the incidence of anti-dsDNA antibodies remained high after the removal of exogenous sources of estrogen, similar to what was observed in estrogen-induced anti-cardiolipin antibodies in C57BL/6J mice (D.Verthelyi, unpublished observations). Interestingly, the incidence of anti-dsDNA antibodies in the control groups increased steadily with age, especially in female mice. Estrogen, therefore, appears to have a long-lasting accelerating and enhancing effect on the expression of these autoantibodies. Studies are being performed on the effects of similar estrogen treatment on mature animals, which may correspond to women of postmenopausal age.

Under normal conditions the synthesis of autoantibodies is down regulated by several mechanisms including clonal deletion and anergy, thus maintaining tolerance of self. There are several potential mechanisms by which estrogen may override tolerance and induce the expression of anti-dsDNA antibodies. First, estrogen may act directly on B

cell populations to override clonal anergy of autoreactive B cells. However, to date, receptors for estrogen on B cells have not been definitively identified. Second, estrogen may act through the hypothalamo-hypophyseal-thymic axis (Grossman et al., 1991) to alter the neuro-immuno-endocrine regulatory circuits. Third, sex hormones may affect other cells of the immune system such as macrophages or T cells to alter the production of cytokines which regulate B cell activity and/or heavy chain isotype switching (e.g. IL-4, IL-5, IL-6, TGF-β; Olsen et al., 1993). Fourth, estrogen may be toxic to cells therefore increasing the exposure to intracellular molecules. Finally, the possibility that estrogen may activate an unknown latent virus or other infectious agents which could promote autoimmunity cannot be ruled out, especially since inoculation with bacterial DNA has been shown to be immunogenic in mice (Glikeson et al., 1991).

Estrogen treatment has been shown to induce antibodies reactive with cardiolipin and other anionic membrane phospholipids in C57BL/6J mice (13,14). Preliminary data suggest that the crossreactivity between estrogen-induced antibodies to dsDNA and cardiolipin is variable and in general low (data not shown). Polyreactive low affinity antibodies and monoreactive autoantibodies could both be induced by estrogen treatment.

There is a vast body of literature which documents that estrogen treatment of normal rodents augments their level of antibodies to many exogenous antigens (Kenny and Gray, 1971; Ansar Ahmed et al., 1985a; Schuurs and Verheul, 1990; Grossman, 1985; Ansar

Ahmed and Talal, 1993; Roubinian et al. 1978). Further, estrogen treatment of autoimmune mice has been shown to promote antibodies to a variety of autoantigens (Ansar Ahmed and Talal, 1991; Roubinian et al., 1978; Brick, 1985). In this study we demonstrated that antibodies to dsDNA can be induced in normal C57BL/6J mice by manipulation of sex steroid levels. It must be emphasized that other than hormonal treatment these mice were not injected with DNA, cardiolipin or any other antigen or preparation from infectious agents. This data together with our previous findings of estrogen induced antibodies to cardiolipin and bromelin-treated erythrocytes supports the concept that estrogen alters immunoregulatory pathways in otherwise normal subjects. This is important since prenatal exposure to diethylstilbestrol, a non-steroidal estrogen, has been shown to enhance the susceptibility to neoplasms and the development of autoimmune disease (Noller et al., 1988; Herbst, 1981). Further, increasing numbers of women are taking estrogen-containing drugs either as contraceptives or as replacement therapies. Therefore it would be of relevance to ascertain whether the exposure to pharmacological levels of estrogen in non-autoimmune individuals (with susceptible genetic backgrounds) could potentially alter their immune competence.

Acknowledgement: This study was supported by a grant from USDA, HATCH program (1-32582) and NIH, BSRG program (4-35032). We thank Dr. Marion for kindly providing supernatants of Monoclonal antibodies to ssDNA and dsDNA, and Dr. Rubin for his input on the performance of the assay. We thank Ms. Betty Mitchell for

performing surgical procedures including hormonal implantation, Mr. Eduardo Romano for his expert assistance in statistical evaluation and Ms. Jane Walsh for her proofing the manuscript.

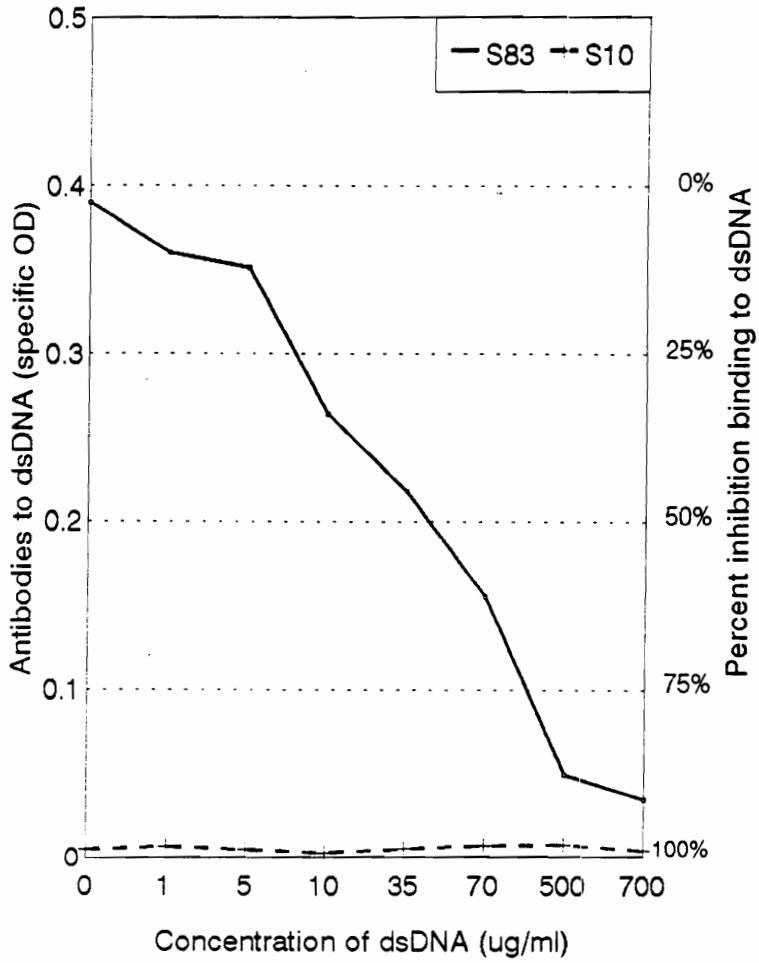


Figure 4.1. Specificity of anti-dsDNA antibody ELISA: To validate the specificity of the anti-dsDNA ELISA, supernatants of cell lines secreting monoclonal antibodies to dsDNA (s83) or to ssDNA (s10) were pre-incubated on plates coated with increasing concentrations of calf thymus dsDNA (0 to 700 ug/ml). This procedure absorbs out antibodies to dsDNA. After incubation at room temperature for two hours, the supernatants were aspirated and tested for anti-dsDNA antibodies as described in the materials and methods section. Note that the reduction in the binding of s83 (specific for dsDNA) is proportional to the concentration of dsDNA in which the supernatant was preincubated. As expected, the binding of s10 (specific for ssDNA) remains at background levels.

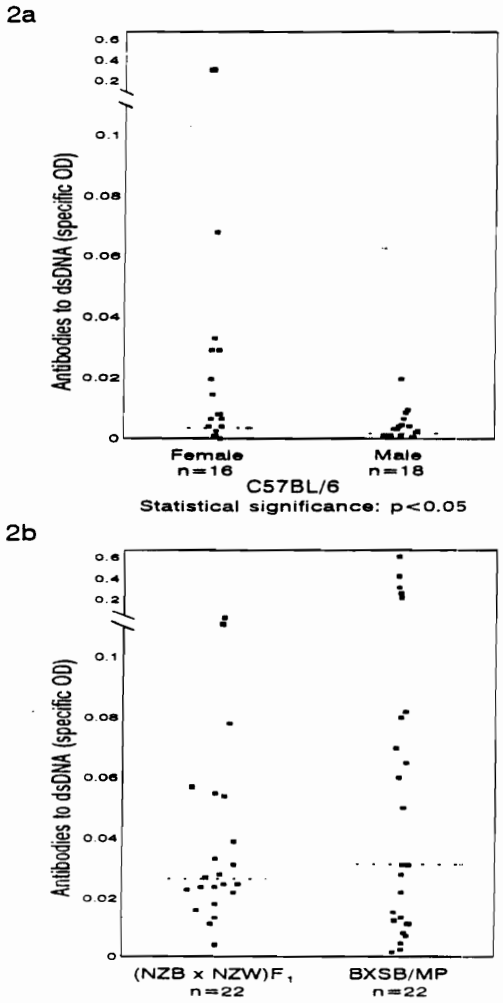


Figure 4.2. Antibodies to dsDNA in C57BL/6J, (NZB x NZW)F_1, and BXSB/MP mice: (a) Male (n=18) and female (n=16) untreated C57BL/6J mice were tested for the expression of antibodies to dsDNA. The figure illustrates the level of anti-dsDNA antibodies of individual mice. The dotted lines represent the median for each group. Differences between male and female C57BL/6 mice were assessed by Mann Whitney test ($p<0.05$). Male mice mean age=10.4 months, range= 4-22 months. Female mice mean age= 10.8 months, range= 3-21 months. (b) Shows the level of antibodies to dsDNA in autoimmune-prone (NZB x NZW) F_1 and BXSB mice as reference.

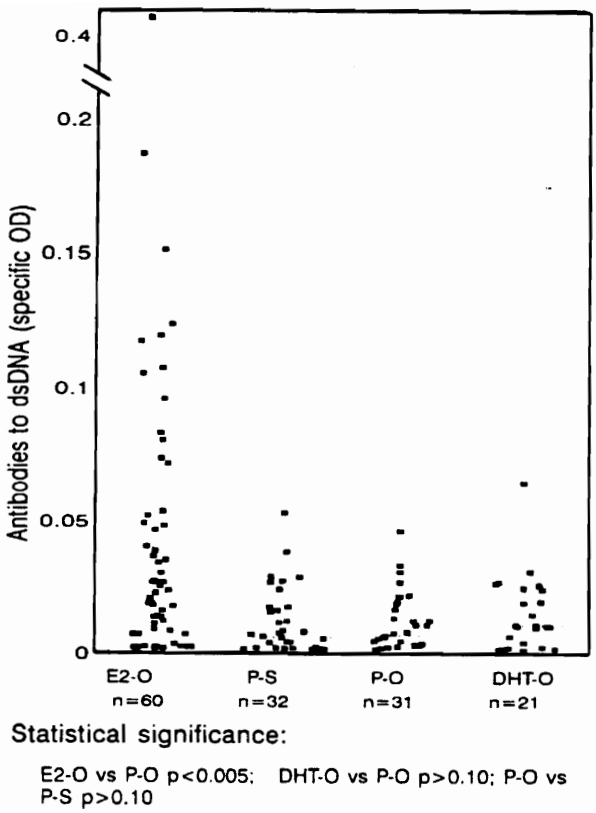


Figure 4.3. Estrogen treatment of orchiectomized C57BL/6J mice induces anti-dsDNA antibodies: Four to five week old prepubertal male C57BL/6J mice underwent orchiectomy and were given 5α-dihydrotestosterone (DHT-Oδ; mean age= 11.3 months, range= 3-13.3 months) or 17β estradiol (E2-Oδ; mean age=7.4 months, range= 3-11.4 months) implants. Control groups include orchiectomized mice with placebo implants (P-Oδ; mean age= 9.5 months, range= 3.3-13.3 months), and sham-orchiectomized mice with placebo implants (P-Sδ; mean age= 8.3 months, range= 3.8-12 months). The figure illustrates the level of IgG anti-dsDNA antibodies of individual mice. The lines represent the median for each group. Data was analyzed by non-parametric Mann-Whitney test.

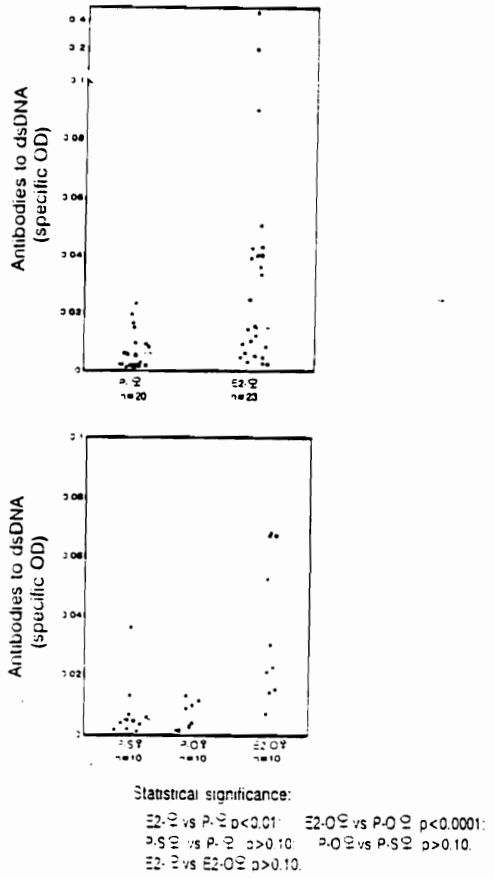
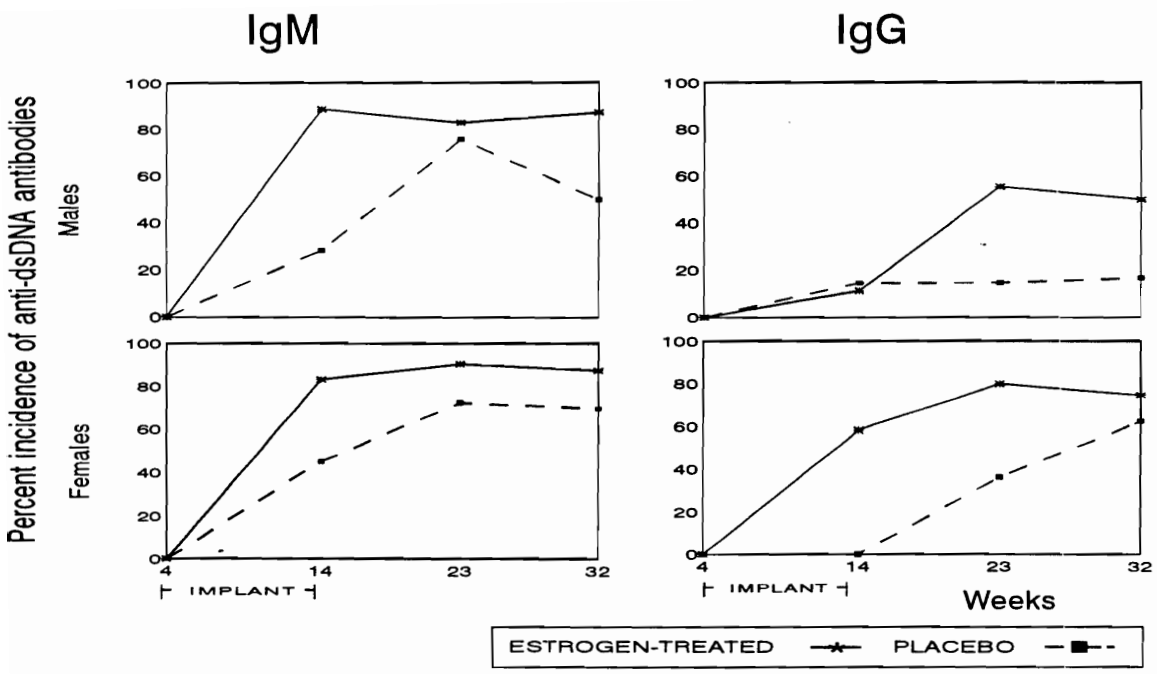


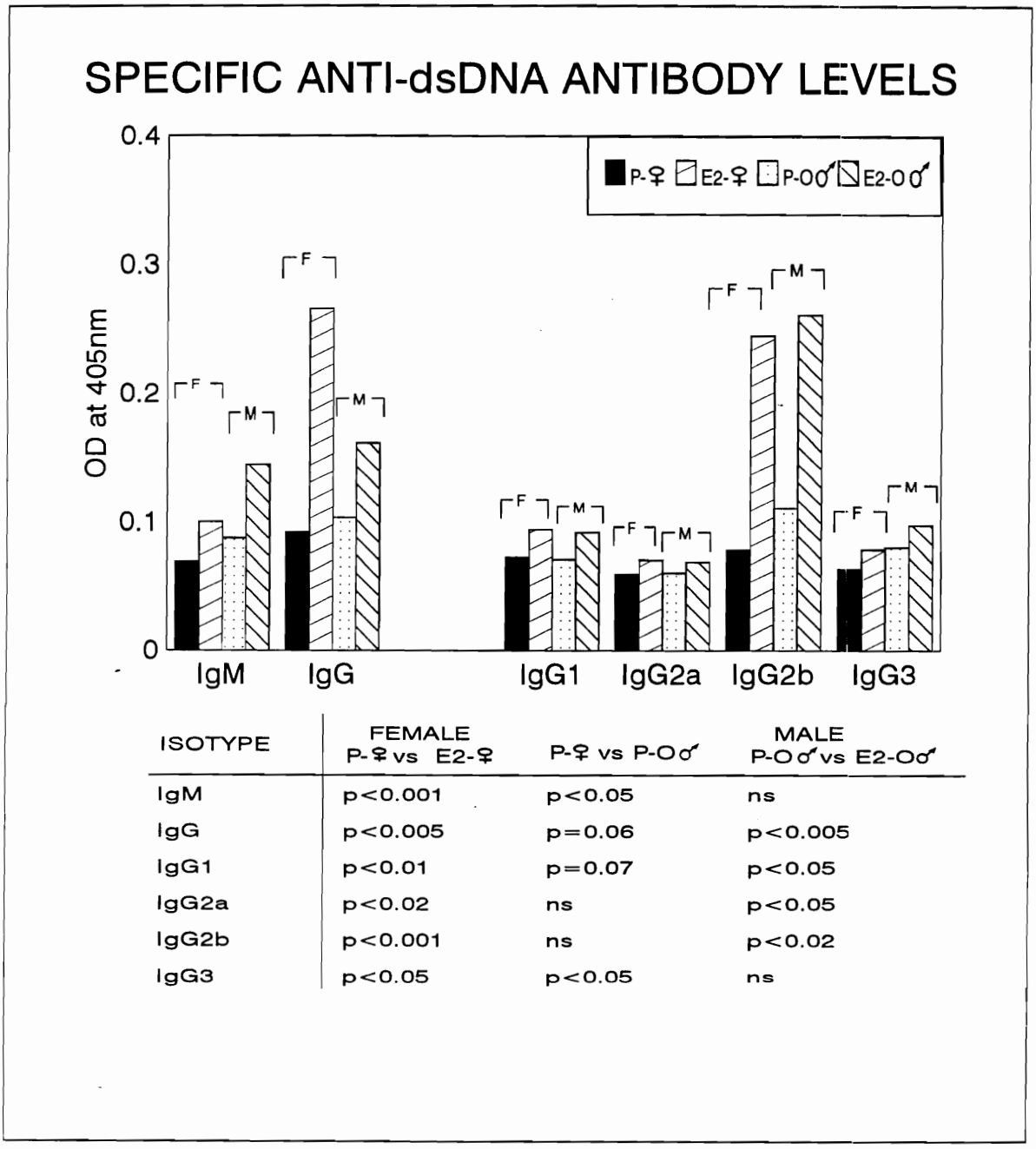
Figure 4.4. Estrogen further increases anti-dsDNA antibodies in female C57BL/6J mice: Four to five week-old female mice were given either 17β-estradiol (E2♀; mean age= 5.7 months, range= 3-7.5 months) or placebo implants (P-♀; mean age= 5.1 months, range= 3-7.8 months). Figure 4.4a shows IgG anti-dsDNA antibodies assessed by ELISA. Note there is a significant increase in the level of anti-dsDNA antibodies in mice treated with estrogen. Figure 4.4b shows the level of anti-dsDNA antibodies in female mice that were ovariectomized and received 17β-estradiol implants (E2-O♀; mean age= 3.5 months, range= 2.5-5.5 months) and their controls [(P-O♀; mean age= 4.2 months, range=2.5-5 months old) and (P-S♀; mean age= 4 months, range= 2.5-5 months)]. The figure illustrates the level of IgG anti-dsDNA antibodies of individual mice. The dotted lines represent the median for each group. Data was analyzed by non-parametric Mann-Whitney test.



Statistical significance:

	IgM			IgG		
WEEK	14	23	32	14	23	32
E2-O♂ vs P-♂	<0.02	NS	NS	NS	<0.01	<0.01
E2-♀ vs P-♀	0.06	NS	NS	<0.005	<0.05	NS

Figure 4.5. Kinetics of estrogen-induced antibodies to dsDNA in (male and female) C57BL/6J mice: Four groups of mice: E2-♀ (n=13), P-♀ (n=13), E2-O♂ (n=9) and P-O♂ (n=9) were tested starting on the day of the implant (4-5 weeks), on the day of the removal of the implant (13-14 weeks) and later on weeks 23, and 32 for anti-dsDNA antibodies. Differences in incidence between E2-O♂ and controls (P-O♂) and between E2-♀ an controls (P-♀) were assessed by Chi square test. (Note that 2 E2-♀, 1 E2-O♂ and 1 P-O♂ died before week 32. Upon postmortem examination , abnormal distension of the bladder was noted in the mice treated with estrogen.)



ISOTYPE	FEMALE P-♀ vs E2-♀	P-♀ vs P-O♂	MALE P-O♂ vs E2-O♂
IgM	p<0.001	p<0.05	ns
IgG	p<0.005	p=0.06	p<0.005
IgG1	p<0.01	p=0.07	p<0.05
IgG2a	p<0.02	ns	p<0.05
IgG2b	p<0.001	ns	p<0.02
IgG3	p<0.05	p<0.05	ns

Figure 4.6. Isotypes of anti-dsDNA antibodies in C57BL/6J mice: Shows the mean level of anti-dsDNA antibodies in the sera from mice of the following groups: P-♀(n=9), E2-♀ (n=8), P-O♂ (n=15) and E2-O♂(n=16). Due to the use of different subisotype-specific antibodies, it is not possible to directly compare among (sub)isotypes. However, it is possible to compare between hormonal treatment groups. Note that almost all IgG anti-dsDNA subisotypes were increased in the sera of estrogen-treated mice.

CHAPTER 5:

CHARACTERIZATION OF ESTROGEN-INDUCED ANTIBODIES TO CARDIOLIPIN IN NORMAL MICE

Verthelyi D. and Ansar Ahmed S. 1996. Characterization of estrogen-induced antibodies to cardiolipin in normal mice. To be submitted to J. Autoimmun.

ABSTRACT:

Antibodies to cardiolipin, in humans, have been associated with a variety of autoimmune disorders including systemic lupus erythematosus, Sjögren syndrome and antiphospholipid syndrome. These antibodies have also been demonstrated in autoimmune-prone MRL-Mp-*lpr/lpr* (MRL/lpr), BXSB-Mp-$^{+yaa}$ (BXSB) and (NZW x BXSB)F_1 (W/B) mice. In previous work, we had shown that male and female nonautoimmune C57BL/6 mice, upon treatment with estrogen, expressed autoantibodies to cardiolipin. In this study, we extend these findings and show that the expression of these antibodies persists for months even after the exposure to exogenous estrogen has been terminated. These antibodies are of IgM and IgG, but not IgA, isotypes, and the predominant IgG subisotype is IgG2b. Estrogen-induced antibodies to cardiolipin only minimally crossreacted with DNA, actin or ovoalbumin.

In humans, the binding of antibodies to cardiolipin from systemic lupus erythematosus patients, has been shown to depend upon the presence of a cofactor, β2-glycoprotein I. We found that in estrogen-treated C57BL/6 mice as well as in SLE-prone MRL/lpr and BXSB mice, anticardiolipin antibodies bind to cardiolipin is the apparent absence of β2-glycoprotein I. Further, addition of exogenous human β2-glycoprotein I to purified immunoglobulin fractions containing anticardiolipin antibodies did not enhance, and at higher concentrations reduced the binding to cardiolipin.

Together, these data show that persistent detectable levels of IgG and IgM autoantibodies specific for cardiolipin can be induced in normal mice by estrogen treatment alone (i.e. without administration of autoantigens). Further, we characterize these antibodies with

regards to their kinetics, crossreactivity, isotype distribution and cofactor (β2-glycoprotein I) requirements, and compare them to autoantibodies from autoimmune prone mice.

INTRODUCTION:

Antibodies to cardiolipin are present in a subset of patients with systemic autoimmune diseases (systemic lupus erythematosus (SLE), Sjögren syndrome (SS), and antiphospholipid syndrome (APS)) (Reviewed in Mcworth-Young, 1990; Lockshin, 1994, Harris, 1990), neurological disorders (focal cerebral ischemia, myelopathy, chorea, migraines, seizures) (Levine and Welch, 1987), and in bacterial (syphilis, borrelia) or viral (HIV-1) infections) (Vaarala et al., 1990; Matsuda et al.,1993; reviewed in Mcworth-Young, 1990; Lockshin, 1994). These antibodies are also induced following the administration of drugs (chlorpromazine and other phenothiazines) (Lillicrap et al., 1990). It is of particular interest that increased antiphospholipid antibodies have been reported in women undergoing *in vitro* fertilization treatment which leads to increased levels of estrogen (Yron et al., 1992; Ben-Chetrit and Ben-Chetrit, 1994).

In mice, spontaneous expression of antibodies to cardiolipin has been reported in some autoimmune-prone strains including MRL/lpr, MRL-Mp-+/+ mice (Gharavi et al, 1989), BXSB (Ansar Ahmed et al., 1993) and W/B mice (Hashimoto et al, 1992), but not in (NZB x NZW)F_1 mice (Gharavi et al, 1989). Antibodies to cardiolipin have been induced in normal mice by administering antibodies to dsDNA carrying the 16/6 idiotype (Blank et al., 1990); and by administration of β2-glycoprotein I alone (Gharavi et al, 1992; Blank et al., 1994) or combined with cardiolipin (reviewed in Triplett, 1992). Antibodies to cardiolipin, however, have not been induced by the administration of cardiolipin alone (reviewed in Triplett, 1992). We have reported that C57BL/6J mice, a nonautoimmune strain, express low levels of autoantibodies to cardiolipin and dsDNA (Ansar Ahmed and Verthelyi, 1993; Ansar Ahmed et al, 1993; Verthelyi and Ansar

Ahmed, 1994). The levels of these autoantibodies are augmented when mice are exposed to exogenous estrogen. In this murine model, the induction of anticardiolipin antibodies does not require the administration of autoantigens.

The *in vitro* binding of antibodies from autoimmune patients to cardiolipin has been shown to depend on the presence of β2-glycoprotein I (McNeil et al., 1990; Galli et al., 1990; Matsuura et al., 1990). This glycoprotein, which is present in human sera (approximately 200 ng/ml), is synthesized in the liver and has been proposed to act by binding to negatively charged molecules such as phospholipids (Wurm et al., 1984). β2-glycoprotein I, has several anticoagulant properties such as the ability to inhibit contact activation of coagulation, adenyldiphosphate (ADP)-induced platelet aggregation, to bind and inhibit activated protein C, and to inhibit the activation of prothrombin (reviewed in Macworth-Young, 1990 and Galli et al., 1993). Antibodies directed to β2-glycoprotein I/ anionic phospholipid complex could interfere with the inhibitory effect of β2-glycoprotein I on procoagulant surfaces. Thus, considerable clinical interest has been raised as to the possibility that the mechanism by which antibodies to cardiolipin facilitate thrombosis is by blocking β2-glycoprotein I activity (Harris and Pierangeli, 1994; Kandiah and Krilis, 1994). Most investigators agree that in SLE and APS patients, the binding of anticardiolipin antibodies to cardiolipin-coated plates is enhanced by β2-glycoprotein I (McNeil et al., 1990; Matsuura et al., 1991; Pierangeli et al., 1992). β2-glycoprotein I-dependent as well as independent anticardiolipin antibodies have been found in women with recurrent fetal loss (Ozawa et al.,1994). Addition of β2-glycoprotein I has been reported to reduce the binding of anticardiolipin antibodies in patients with hemophilia (Matsuda et al., 1993) syphilis and HIV (Matsuura et al., 1991; Hunt et al., 1992; Gharavi et al., 1994), as well as in patients with repeated abortions

(Ozawa et al., 1994). However, whether antibodies to cardiolipin bind directly to cardiolipin, to a cryptic epitope of cardiolipin exposed by the presence of β2-glycoprotein I, or to a new epitope that is formed/exposed when cardiolipin and β2-glycoprotein I combine, is still a controversy (Harris and Pierangeli 1994; Wagenknecht and McIntyre, 1993; Roubey, 1994). Most (Matsuura et al., 1992; Pierangelli et al., 1992; Gharavi et al., 1993), but not all (Galli et al., 1990; Roubey et al, 1995), researchers agree that antibodies to cardiolipin do not bind to β2-glycoprotein I alone.

In this study we characterize the antibodies to cardiolipin elicited in nonautoimmune C57BL/6 mice by estrogen treatment with regard to their kinetics, crossreactivity, and isotype and subisotype distribution. Further, we investigate whether the binding of antibodies to cardiolipin from estrogen-treated normal mice estrogen requires the presence of cofactor β2-glycoprotein I. This aspect was compared with autoimmune-prone MRL/lpr and BXSB mice.

MATERIALS AND METHODS:

Mice: C57BL/6J and MRL/lpr mice were purchased from the Jackson Laboratories (Bar Harbour, Maine). Mice were fed on a commercial diet, and housed in standard cages (3 to 5 mice/cage) with a 14 light/10 dark hours schedule.

Sex hormonal treatment: Four to five-week-old mice were orchiectomized under pentobarbital anesthesia as described previously (Ansar Ahmed et al., 1986; Ansar Ahmed et al., 1989). At this time, silastic implants (Dow Corning Co., MI) or commercial pellets (Innovative Research of America, Toledo, OH) containing 17β-

estradiol, were surgically placed subcutaneously as reported earlier (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994). Mice with gonadectomies or sham gonadectomies, that received placebo implants served as controls. The commercial pellets, which were used in the sequential studies, are time-release capsules designed to slowly release sex hormones for 2-3 months. Silastic implants [(7mm long, containing 4-6 mg of 17β-estradiol (Sigma, St.Louis, MO)] also release hormones slowly for several months (Ansar Ahmed et al., 1989). In previous studies, we found no significant difference between the two types of estrogen preparations in terms of induction of anticardiolipin antibodies (Ansar Ahmed and Verthelyi, 1993) or their modulation of the T cell proliferative response (Ansar Ahmed, unpublished observations). The levels of estradiol in pooled sera from 3-6 mice per each hormonal treatment group were assessed by the radioimmunoassay performed by Dr T.J. Reimer (Diagnostic Laboratory, New York College of Veterinary Medicine, NY). As reported previously [14], we found that estrogen levels were three times higher in estrogen-treated mice than in the placebo-treated controls. The levels of estrogen comparable were comparable to those in pregnant mice (reviewed in Beamer et al., 1983).

Serum collection: Serum from each mouse was collected retroorbitally and kept at -70°C until tested. Frozen sera from BXSB mice were obtained from the laboratory of Dr. V. Dennenberg, University of Connecticut.

Antigens and antibodies : Purified human β2-glycoprotein I was obtained from 3 different sources: Dr Matsuura, Immunology Laboratory, Diagnostic Division, Yamasa Corp. Choshi-shi, Japan; Celsus laboratories (Cincinnati, OH), and Dr Alice Gilmann Sachs, Chicago Medical Hospital, Chicago, Illinois. Monoclonal antibodies to

cardiolipin, WB-CAL-1, derived from W/B mice, which have increased binding to cardiolipin in the presence of β2-glycoprotein I, were kindly provided by Dr Matsuura and Dr.Koike, Hokkaido University School of Medicine, Sapporo, Japan.

Purification Immunoglobulin fraction: The immunoglobulin fraction (Ig-fraction) of murine sera was purified utilizing EZ-sep kit (Pharmacia, Piscataway, NJ) as per manufacturer's instructions. The immunoglobulin fractions were then resuspended in PBS to the original serum volume. The purity of the immunoglobulin fraction was assessed by protein electrophoresis; the gels were stained with silver as per manufacturers's instructions. Since the molecular weight of β2-glycoprotein I is similar to that of the heavy chain of IgG, 12% native acrylamide-bisacrylamide gels were utilized to prevent the reduction of the disulfide bonds of immunoglobulins. By this method, β2-glycoprotein I was not readily apparent in the immunoglobulin fraction (**Figure 5.1**). The purified immunoglobulin-fractions were tested for antibodies to cardiolipin at a 1:100 dilution.

ELISA assays: Antibodies to cardiolipin were assessed by standard ELISA as described previously (Ansar Ahmed and Verthelyi, 1993). Briefly, medium binding 96-well flat bottom plates (Costar, Cambridge, MA) were coated with 30 μl/well (50 μg/ml of cardiolipin in ethanol) and left uncovered overnight in the dark at 4°C. After washing with PBS, the wells were blocked with PBS containing 2% bovine serum albumin (BSA; 75 μl/well) for 90 minutes. The wells were washed three times with PBS. In initial experiments (sequential studies), serum samples were diluted in PBS containing 10% heat-inactivated adult bovine serum (PBS-10% ABS, JRH Bioscience, KS). In all subsequent studies, the sera were diluted in PBS-1% BSA to avoid the potential

interference of bovine β2-glycoprotein I (which is thought to be present in ABS). The serum samples (1:100 dilution) were incubated in the wells for 3 hours at room temperature. After washing, 50 ul of alkaline phosphatase conjugated goat anti-mouse heavy-chain specific immunoglobulin were added and the plates were further incubated for 1 hour at 37°C in a humid chamber (enzyme-conjugated antibodies from Caltag, South San Francisco, CA, were used in sequential studies; antibodies from Southern Biotechnologies Assoc. were utilized in all other studies). Plates were washed again and then developed with p-nitrophenylphosphate (Sigma, MO) in diethanolamine buffer (pH 9.8), and read after 60 minutes (405 nm, Molecular Devices, Menlo Park, CA). Negative controls included wells with known negative sera, and wells with no sera ("no serum blanks"). The absorbencies of wells containing known negative sera were similar to that of "no serum blanks", therefore, "no serum blanks" were utilized to control for background. In addition, known positive sera from MRL/lpr mice were included on each plate to monitor the validity of the assay. Antibody levels are reported as specific optical density (Specific OD), which were obtained by subtracting the mean OD of the "no serum blanks" on the plate from the mean OD of the sample (Ansar Ahmed et al., 1993; Verthelyi and Ansar Ahmed 1994).

In experiments to assess the specificity of estrogen-induced anticardiolipin antibodies, sera were preincubated in wells coated with cardiolipin (0.5 mg/ml in absolute alcohol) or alcohol alone for 3 hours at room temperature as described previously (Ansar Ahmed et al., 1993). Sera were subsequently tested for the presence of antibodies to different antigens.

In experiments designed to determine the role of β2-glycoprotein I as a cofactor for the

binding of anticardiolipin antibodies to cardiolipin, care was taken to avoid potential sources of β2-glycoprotein I (eg. ABS). Therefore, the blocking was performed utilizing PBS containing 2% BSA plus, while the sample and the secondary antibodies were diluted in PBS containing 1% BSA and 0.3% gelatin. Two different protocols were utilized to assess the effect of addition of exogenous β2-glycoprotein I with similar results. In the first method, purified human β2-glycoprotein I (0-32 μg/ml) was coated onto cardiolipin coated plates diluted in HEPES 1% BSA overnight at 4°C. The wells were then blocked and the anticardiolipin assay was performed as described above. In the second protocol, cardiolipin-coated wells were blocked, washed, and then β2-glycoprotein I in concentrations similar to the ones stated above was added to the wells. After incubating the β2-glycoprotein I in the wells for 10 minutes at room temperature the samples were added (sera or purified Immunoglobulin-fraction of sera) to the wells (as described in Matsuura et al., 1992). In both protocols, the final dilution of the sera was 1:200 to reduce the potential influence of endogenous β2-glycoprotein I in the serum sample.

Statistical Analysis: Chi-Square test was used to determine the significance of differences in the incidence of antibodies between groups. Non-parametric ANOVA or Mann-Whitney tests were employed to test for differences in the level of antibodies between groups. In the experiments to assess the effect of β2-glycoprotein on the binding of anticardiolipin antibodies, the data was analyzed by calculating the quadratic relationship of the cofactor concentration and absorbance for each individual sample. The distribution and slope of each term of the quadratic function was evaluated utilizing the univariate test in SAS. Student "t" tests were used to test whether the mean coefficients were different form "0".

RESULTS:

Estrogen accelerates the expression of antibodies to cardiolipin: Kinetic studies were performed to assess whether the effects of estrogen on anticardiolipin antibodies was transient or long lasting. Eighteen 4-5 week-old male C57BL/6 mice (prepubertal) were orchiectomized and implanted with either estrogen (n=9) or placebo (n=9) pellets. Four to five week-old female mice (13 per group) received either placebo or 17β-estradiol implants. Estrogen implants were surgically removed ten weeks post-implantation to ensure that mice would not be exposed to more exogenous estrogen. Placebo implants were also removed as a control for surgical stress. The presence of antibodies to cardiolipin (IgG, IgM and IgA) in sera was measured at the time of administration of the implant, upon removal of the implant (13-14 weeks), and at 23 and 32 weeks of age. As shown in **Figure 5.2** anticardiolipin antibodies were not detectable at the time of administration of the estrogen/placebo implants. There was a steady increase in the proportion of estrogen-treated males that expressed IgG anticardiolipin antibodies. The incidence of these autoantibodies continued to increase for several weeks after the exposure to estrogen had been terminated. Male C57BL/6 mice that had received placebo treatment exhibited a low incidence of IgG antibodies to cardiolipin throughout the study. Female mice (with intact ovaries) treated with placebo showed a slow, age-dependent, increase in the incidence of IgG antibodies to cardiolipin. Estrogen-treated females had an accelerated and enhanced expression of these antibodies.

The incidence of antibodies to cardiolipin of IgM isotype appeared to increase with age in both sexes. Estrogen-treated female mice also showed increased and accelerated expression of IgM antibodies to cardiolipin. In males, however, differences were

unremarkable. IgA antibodies to cardiolipin were not detectable in any of the treatment groups.

Estrogen-induced antibodies to cardiolipin show only marginal crossreactivity with other antigens. To determine whether the anti-cardiolipin antibodies elicited by estrogen were specific for cardiolipin, inhibition studies were carried out. Sera from estrogen-treated mice with disparate levels of antibodies to cardiolipin were incubated in cardiolipin-coated wells for 3 hours and subsequently tested for antibodies to this and other antigenic specificities. As shown in **Figure 5.3**, the binding to cardiolipin was reduced by 66%, while the binding to the other antigens was only reduced by about 20% ($p < 0.001$). Importantly, incubating sera in uncoated wells reduced the subsequent binding to all antigens tested by 15 to 23%. These results indicate that these antibodies are specific for cardiolipin.

Analysis of isotype and subisotype of anticardiolipin antibodies in estrogen-treated mice: Antibodies that bound to cardiolipin in estrogen-treated C57BL/6 mice were of IgM and IgG isotype. IgA antibodies were not detectable. Analysis of IgG subisotypes revealed that IgG1, IgG2b, IgG3, and IgG2a anticardiolipin antibodies were all detectable. It was not possible to directly compare between the levels of the different isotypes and subisotypes of anticardiolipin-specific antibodies because isotypic/subisotypic-specific commercial reagents of differing degrees of sensitivity were employed. Nevertheless, it was possible to compare the level of antibodies of estrogen-treated and placebo-treated mice for each individual isotype and subisotype. As shown in **Figure 5.4,** estrogen-treated male mice had a significant increase in the levels of anticardiolipin antibodies of IgM ($p < 0.01$) and IgG isotypes ($p < 0.001$). Analysis of the

subisotypes revealed that estrogen-induced antibodies to cardiolipin were mainly of IgG2b ($p < 0.001$), and to lesser extent of IgG1 ($p < 0.05$) and IgG3 ($p < 0.05$) subisotypes.

The binding of anticardiolipin antibodies to cardiolipin is not enhanced by the presence of purified human β2-glycoprotein I. In humans, β2-glycoprotein I has been shown to increase the binding of anticardiolipin antibodies from autoimmune patients onto cardiolipin-coated ELISA plates. We had previously reported that the addition of increasing concentrations of adult bovine serum which presumably contains β2-glycoprotein I (Matsuura et al., 1991), did not enhance, but rather reduced the binding of anticardiolipin antibodies from C57BL/6 mice (Ansar Ahmed and Verthelyi, 1993). To further study the role of β2-glycoprotein I in the binding of murine anticardiolipin antibodies to cardiolipin, we carried out a modified ELISA. β2-glycoprotein I, at increasing concentrations (0-30 μg/ml) was incubated in the cardiolipin-coated wells for 10 minutes and then the serum samples (1:200 dilution) were added and incubated for three hours (as reported by Matsuura et al., 1992). β2-glycoprotein I of human origin was chosen since it has a higher sequence homology to murine β2-glycoprotein I (which is not commercially available) than bovine-derived β2-glycoprotein I (Sellar, 1994). **Figure 5.5a** shows that at higher concentrations of β2-glycoprotein I did not enhance, but rather reduced the binding of anticardiolipin antibodies from estrogen-treated C57BL/6 mice. Similar results were obtained with sera from MRL/lpr and BXSB mice (**Figure 5.5b&c**). Statistical analysis of the quadratic relationship between β2-glycoprotein I concentration and the anticardiolipin antibody binding to cardiolipin of each individual sera revealed that the binding of the anticardiolipin antibodies was significantly reduced ($p < 0.001$) by co-incubation with increasing concentrations of the cofactor. The lack of positive effect of β2-glycoprotein I in the binding of anticardiolipin

antibodies to cardiolipin was confirmed utilizing β2-glycoprotein I from three different sources as well as another experimental protocol which involved postcoating of β2-glycoprotein I on cardiolipin-coated wells overnight at 4°C (data not shown).

Antibodies to cardiolipin in sera of MRL/lpr and C57BL/6 mice bind to cardiolipin in the absence of β2-glycoprotein I.

Estrogen has been shown to modulate the hepatic function (Fahim et al., 1971). Since estrogen could potentially alter the endogenous levels of β2-glycoprotein I by modifying its rate of synthesis and/or degradation, it was important to rule out the possibility that the estrogen-induced increase in anticardiolipin antibodies was a reflection of augmented levels of β2-glycoprotein I in sera. Utilizing an EZ-sep kit from Pharmacia, the immunoglobulin fraction from sera of estrogen-treated C57BL/6 and MRL/lpr mice was isolated. As shown in **figure 5.6**, the level of anticardiolipin antibodies that bind to plates coated with cardiolipin alone in the immunoglobulin fractions (which did not have detectable levels of β2-glycoprotein I) was comparable to that of whole serum, thus reinforcing the notion that endogenous β2-glycoprotein is not modifying the binding of anticardiolipin antibodies in these mice.

Addition of β2-glycoprotein I does not enhance the binding of purified immunoglobulins to cardiolipin. As had been observed with sera, addition of increasing amounts (0-30 μg/ml) of human β2-glycoprotein I to the immunoglobulin-enriched fractions did not enhance, and in most mice reduced, the binding of anticardiolipin antibodies (**figure 5.7**), in estrogen-treated C57BL/6 as well as autoimmune prone MRL/lpr and BXSB mice. In this experiment we had included a

monoclonal antibody derived from (W/B) F_1 mice (WB-CAL 1), which had previously been shown to have increased binding in the presence of human β2-glycoprotein I as a control (Hashimoto et al., 1992). We found that the binding of this monoclonal antibody was enhanced by the addition of increasing concentrations of β2-glycoprotein I as had been reported (Hashimoto et al., 1992). As had occurred with sera, the enriched Ig-fractions did not show an increase -rather a decrease- when tested for anticardiolipin antibodies in cardiolipin-coated wells that had been postcoated overnight with increasing concentrations of β2-glycoprotein I (data not shown).

DISCUSSION

A large percentage of the human population is exposed to exogenous estrogen. Many women utilize estrogen-based oral contraceptives for most of their reproductive life, and in recent years estrogen replacement for post menopausal women has become a common practice. Further, it is now recognized that a number of insecticides, pesticides and industrial waste products (2,4-D, Dioxin, DDT, to name a few), that are released daily into the environment can disrupt the hormonal status in animals and humans (reviewed in Colburn et al., 1993).

A close interaction between the immune and endocrine systems is now well recognized (Ansar Ahmed et al., 1985; Schuurs and Verheul, 1990). Sex hormones have been shown to affect not only the reproductive tissues, but non-classic targets such as the bone marrow and the thymus as well. Although a number of studies have examined the effects of estrogens on the immune system of autoimmune-prone mice, there is little information on their role as modulators of the immune function in normal mice. We have shown in

previous studies that exposure of nonautoimmune C57BL/6 mice to estrogen induced the expression of autoantibodies to cardiolipin and other anionic phospholipids (Ansar Ahmed and Verthelyi, 1993), and to dsDNA (Verthelyi and Ansar Ahmed, 1994). In the present study we show that the effect of estrogen on the immune system is long lasting. Similar to what was observed for estrogen-induced antibodies to dsDNA, the expression of antibodies to cardiolipin persists for months after the exposure to exogenous estrogen has been terminated. It must be emphasized that other than the hormonal manipulation these mice were not deliberately inoculated or stimulated in any way.

Recent studies had shown that estrogen induces antibodies to a wide variety of auto and heteroantigens (Verthelyi and Ansar Ahmed, 1996), and that the estrogen-induced antibodies to cardiolipin crossreact with other anionic, but not zwitterionic membrane phospholipids (Ansar Ahmed et al, 1993). In humans, antibodies to cardiolipin have occasionally been reported to be crossreact with dsDNA (Shoenfeld et al., 1983); this is thought to be due to some structural similarities (Lafer et al., 1981). In this study, we show that the anticardiolipin antibodies expressed in estrogen-treated nonautoimmune mice do not crossreact significantly with other antigens, including dsDNA.

Anticardiolipin antibodies of IgM, IgG and IgA isotypes have been found in SLE and syphilis patients. Reports of anticardiolipin antibody subisotype distribution in SLE patients differ; IgG1 and IgG3 (Tsutsumi et al., 1988; Loizou et a., 1992) or IgG2 and IgG4 (Levy et al., 1990) have been reported to be predominant. In syphilis patients, antibodies to cardiolipin are mainly of IgG1, IgG3 and IgG4 subisotypes (Loizou et al.,1992). In mice, the MRL/lpr strain express IgG, IgM and IgA anticardiolipin antibodies, and the predominant IgG subisotypes are IgG2a and IgG2b (Gharavi et al.,

1989). The estrogen-induced anticardiolipin antibodies in C57BL/6 mice are of IgM and IgG, but not IgA, isotype. IgG subisotype analysis of these antibodies indicate that estrogen-induced anticardiolipin antibodies belong to IgG2b, IgG1 and IgG3 subisotypes, however, subisotype IgG2b is predominant. Similarly, in BALB/c mice treated with estrogen we find that the principal IgG subisotypes of antibodies to cardiolipin are IgG1 and IgG2b, while those elicited by vaccination with *Brucella abortus*, strain RB51, are of IgG2a, IgG2b and IgG3 subisotypes (D. Verthelyi, G. Schurig and Ansar Ahmed, unpublished observations). TGF-β_1 has been reported to promote isotype switch to IgG2b (McIntyre et al., 1993); however, we found that sera of male and female estrogen-treated mice had reduced levels of TGF-β_1 compared to controls (data not shown). The possibility that TGF-β-like molecules, akin to those found in synovial fluid of RA patients (Abedi-Valugerdi et al., 1993), may be involved in the isotype switch to IgG2b needs to be examined.

There are several potential mechanisms by which estrogen may increase the production of IgG antibodies to cardiolipin: First, estrogen may act directly on B cell populations. However, to date, receptors for estrogen on B cells have not been definitively identified. Second, estrogen may act through the hypothalami-hypophyseal-thymic axis to alter the neuro-immuno-endocrine regulatory circuits (Grossman, 1991). Third, estrogen may affect B cells by down regulating other immune cells such as suppressor T cells (Paavonen, 1981; Ansar Ahmed et al., 1985b; Clerici et al., 1991) or NK cells (Seaman et al., 1978; Nilsson and Carlsten, 1994), or by altering the production of cytokines (eg. IL-4, IL-5, IL-6, TGF-β) which regulate B cell activity (Olsen et al.,1993; Rook et al., 1994). Fourth, estrogen might be increasing the availability of phospholipids to the immune system though direct cellular toxicity or by boosting cell activation. Cell

activation has been shown to modify the phospholipid asymmetric distribution on the cell membrane and lead to increased phosphatidylserine on the external phase of the cell membrane (reviewed in Bevers et al.,1994). By facilitating the activation of certain cells, estrogen could augment the availability of phospholipids on the cell surface to the immune system. And, finally, administration of estrogen has been reported to reduce the bone marrow cellularity (Seaman et al., 1978; Ansar Ahmed et al., 1985b). The increase in local cell death may provide a source of intracellular antigens. Despite the evidence that estrogen treatment induces an increase in immunoglobulin levels, it is interesting to note that estrogen has been reported to induce a decrease in B cell precursors at the bone marrow level (reviewed in Kincade et al., 1994). We have found that estrogen-treated mice develop hematopoietic centers in spleen and liver (D. Verthelyi, A. Saunders and Ansar Ahmed, unpublished observations). Autoimmune B cell clones could potentially derive from B-cell hematopoiesis in tissues other than bone marrow.

In humans, the presence of IgG antibodies to cardiolipin has been associated with increased thrombosis, thrombocytopenia, and recurrent fetal loss. There is some evidence to suggest that anticardiolipin antibodies have a pathogenic effect on mice. For example, high levels of antibodies to cardiolipin have been reported in (W/B) F1 mice, which have an unusually high incidence of myocardial infarcts and thromboembolic disorders (Hashimoto et al., 1992). Also, active and passive expression of anticardiolipin antibodies in nonautoimmune BALB/c and ICR mice resulted in reduced fecundity rate, increased resorption index of embryos per pregnancy, and reduced embryo and placental weights (Blank et al., 1991; Blank et al., 1994; Piona et al, 1995). These effects, however, were prevented if the mice were treated with aspirin, or heparin (reviewed in

Lockshin, 1994) suggesting that the putative pathogenic role of these antibodies may be linked to their ability to disrupt the anticoagulant properties of β2-glycoprotein I. We have not yet established the pathogenic role of estrogen-induced anticardiolipin antibodies in mice. This study, however, appears to indicate that their binding is not dependent upon the presence of β2-glycoprotein I. Further, the addition of exogenous β2-glycoprotein I (both in solution as well as on solid phase) particularly at higher doses reduced, rather than enhanced the binding of anticardiolipin antibodies to cardiolipin-coated plates. Similar results were observed for antibodies to cardiolipin in BALB/c mice that were induced by exposure to estrogen or to *Brucella abortus* strain RB51 (D. Verthelyi, unpublished observations). It is important to point out that the β2-glycoprotein I utilized in these studies was of human origin. Human β2-glycoprotein I has, however, been shown to enhance the binding of some but not all monoclonal antibodies to cardiolipin derived from (W/B)F_1 mice (Hashimoto et al., 1992). Importantly, as reported earlier, addition of human β2-glycoprotein I increased the binding of (W/B)F_1-derived monoclonal antibody WB-CAL 1, thus suggesting that the lack of cofactor activity estrogen-treated C57BL/6 mice is not due to the human source of β2-glycoprotein I or to subtle methodological differences. We also report here that the addition of β2-glycoprotein I did not enhance the binding of anticardiolipin antibodies from autoimmune-prone MRL/lpr and the BXSB mice, which are established models for SLE. The lack of β2-glycoprotein I-dependence in the binding of MRL/lpr anticardiolipin antibodies has also been discussed in a previous study (Hashimoto et al., 1992). The reduction in the binding observed when human β2-glycoprotein I was added suggests that the epitope recognized by the murine anticardiolipin antibodies could be partially blocked by the presence of human β2-glycoprotein I.

These studies confirm that estrogen can influence the immune system to allow the expression of IgM and IgG autoantibodies specific for cardiolipin in normal C57BL/6 mice. Importantly, the expression of these autoantibodies persisted for months after the estrogen had been removed. Interestingly, unlike what has been described in human SLE patients, the binding of murine anticardiolipin autoantibodies from estrogen-treated nonautoimmune mice as well as from autoimmune-prone mice appeared to be independent of the presence of β2-glycoprotein I, and addition of exogenous human β2-glycoprotein I reduced, rather than enhanced, their binding. Studies utilizing murine β2-glycoprotein I, which is currently not commercially available, are needed to conclusively determine the role of β2-glycoprotein I in the binding of antibodies to cardiolipin in mice.

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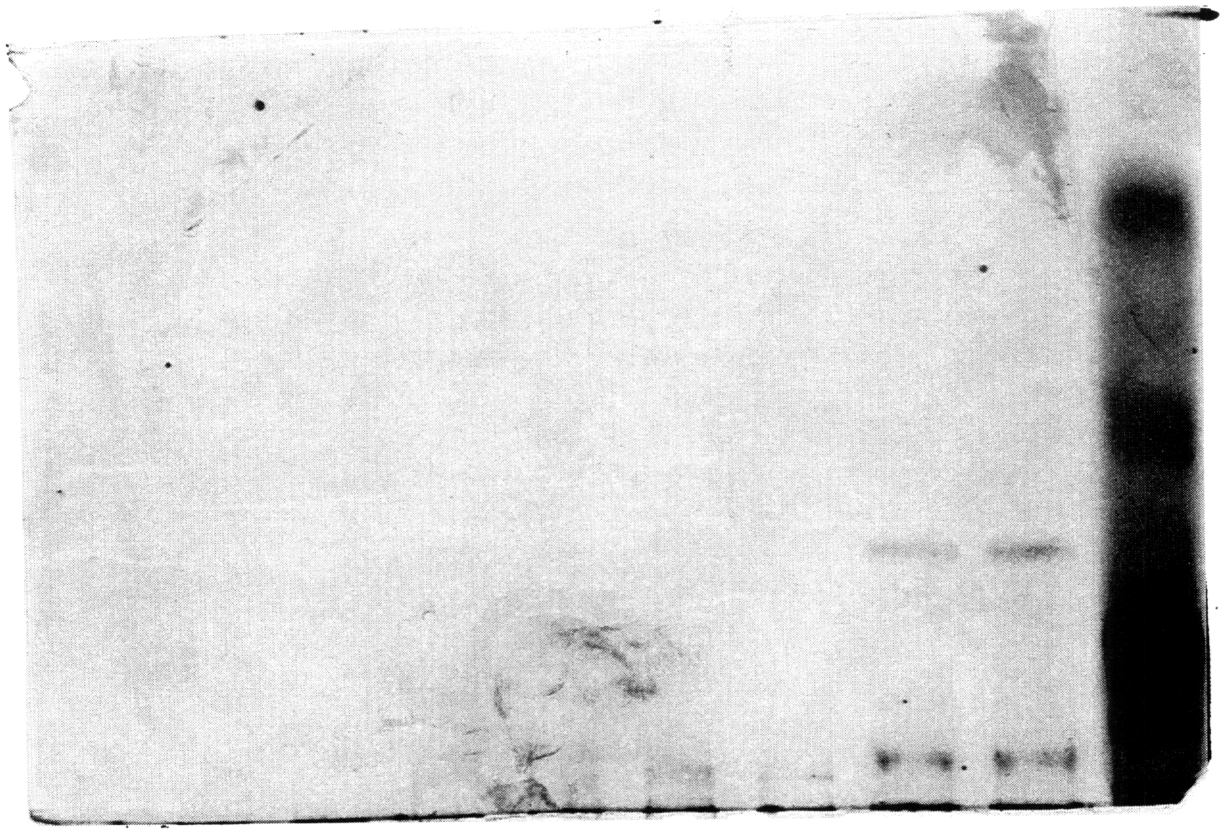


Figure 5.1: Gel electrophoresis stained with silver as per manufacturer's instructions (Biorad). lane 1 and 2: 150 ng of human β2-glycoprotein I; lane 3: purified murine IgG monoclonal antibody; lanes 4, 5 and 6: purified Ig fractions (approximately 2.5 μg of protein per lane) of C57BL/6 , MRL/lpr and BXSB sera respectively; lane 9: purified murine IgM.

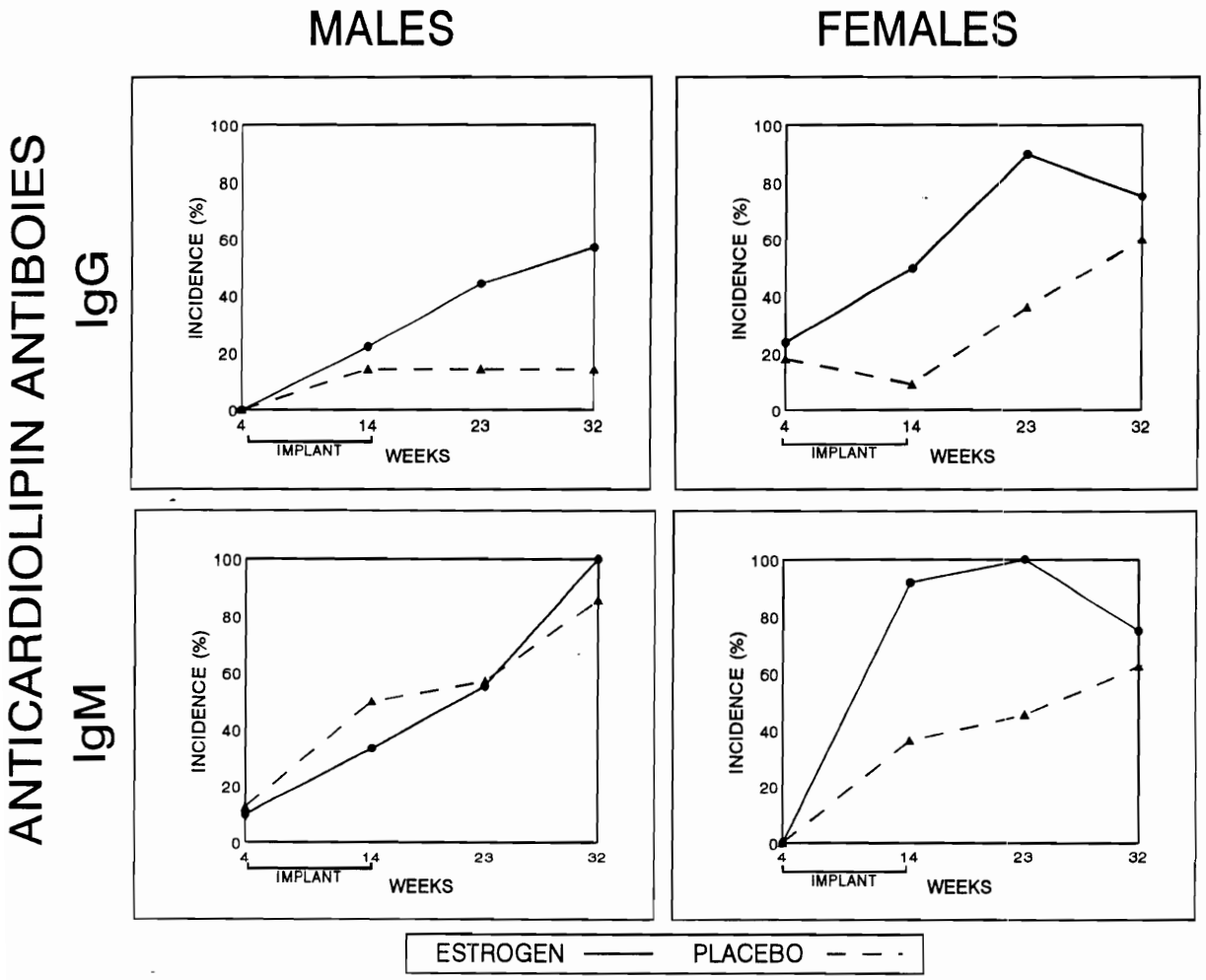


Figure 5.2: Male (n=9 per group) and female (n=13 per group) C57BL/6 prepubertal mice were treated with estrogen or placebo implants. Male mice were orchiectomized. Mice were tested for anticardiolipin antibodies in sera on the day the implant was inserted (4-5 weeks), on the day of the removal of the implant (13-14 weeks) and later on weeks 23 and 32. Significance of differences in the incidence between estrogen-treated and control mice were assessed by the Chi square test. (Note that 3 mice treated with estrogen, 2 females and 1 male, and 1 male mouse treated with placebo died before week 32).

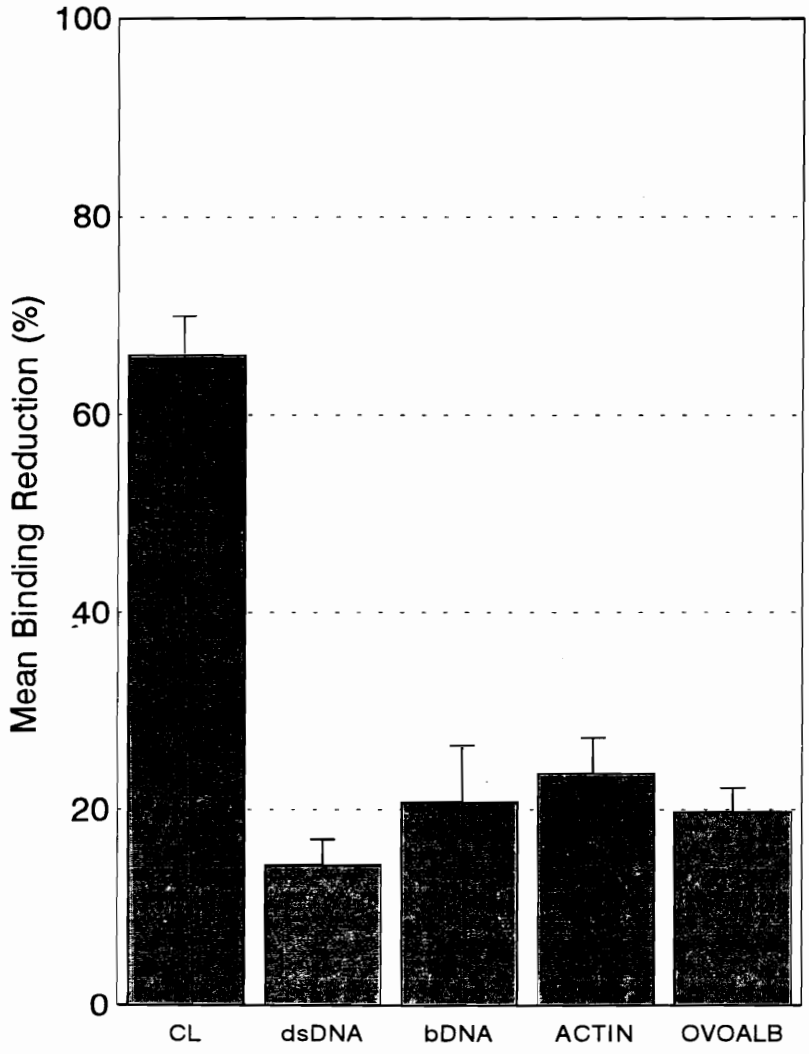


Figure 5.3: Eight sera from estrogen-treated male C57BL/6 mice that had IgG antibodies to cardiolipin, double-stranded DNA (dsDNA), bacterial DNA (bDNA), actin or ovoalbumin (OVOALB.) were incubated in cardiolipin-coated wells for 3 hours at room temperature and subsequently tested for the presence of antibodies to all 5 antigens. The reduction in the binding of antibodies to cardiolipin was significantly higher compared to the other antigens as assessed by ANOVA ($p < 0.001$). The graph depicts the mean $\pm$ SEM reduction in the binding.

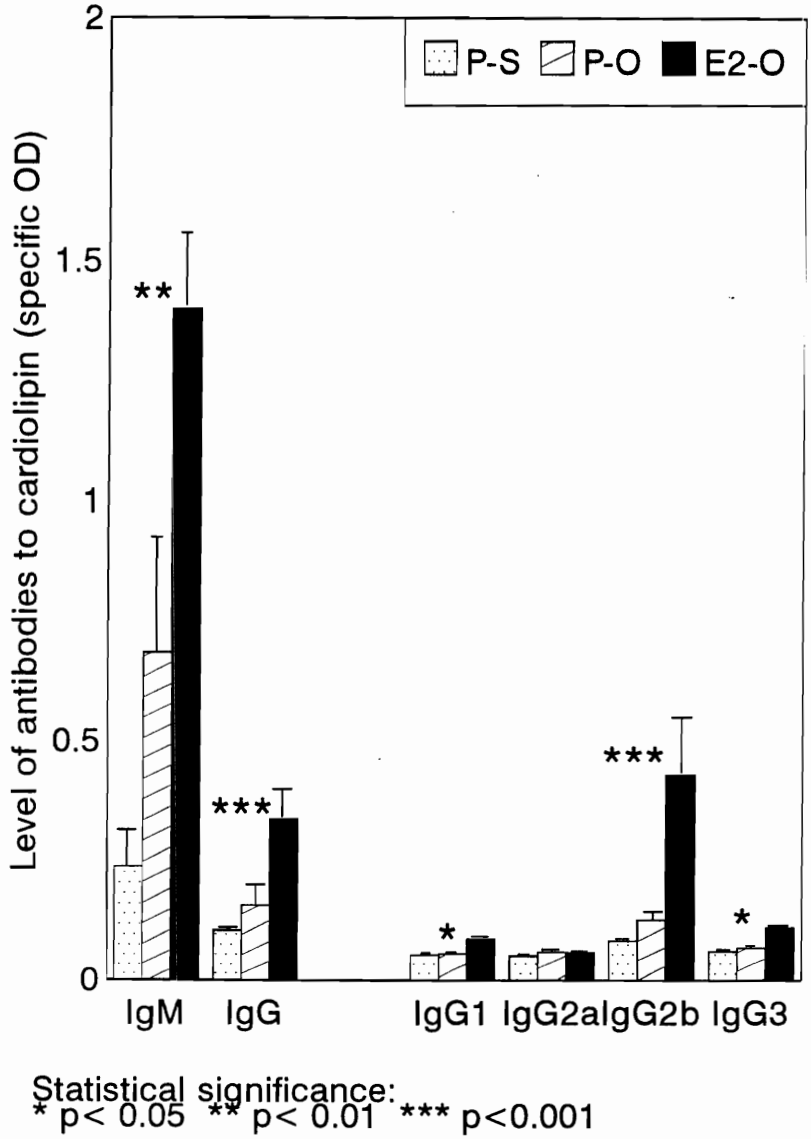


Figure 5.4: Shows the mean ± SEM isotype and subisotype levels of anticardiolipin antibodies in sera from sham-orchiectomized and orchiectomized males that received placebos [P-S (n=10), and P-O (n=10), respectively) and orchiectomized males that received estrogen implants (E2-O, n=13). Due to the use of different subisotype-specific antibodies, it was not possible to directly compare among (sub)isotypes. However, it is possible to compare between hormonal treatment groups. Statistical differences were assessed using a three-way Kruskal-Wallis test.

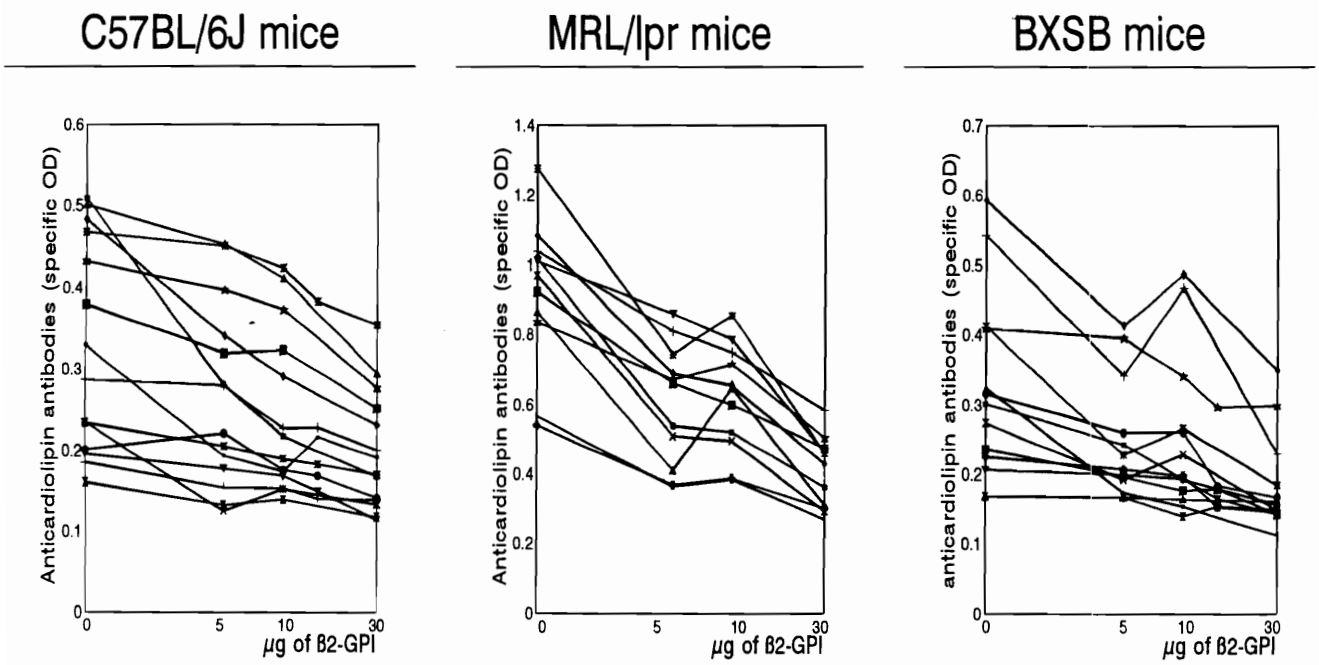


Figure 5.5: Shows the binding of anticardiolipin antibodies from individual male mice to cardiolipin coated plates in the absence or presence of increasing concentrations of human β2-glycoprotein I (β2-GPI). The β2-glycoprotein I was incubated in the wells for 10 minutes prior to the addition of sera. The distribution of the individual quadratic relationship between cofactor concentration and absorbance was tested using an univariate analysis. For all 3 strains (estrogen-treated C57BL/6, MRL/lpr and BXSB mice) the linear regression coefficients were significantly different from 0 ($p < 0.01$) and the slopes were negative indicating that increasing concentrations of β2-glycoprotein I resulted in a reduction of the absorbance.

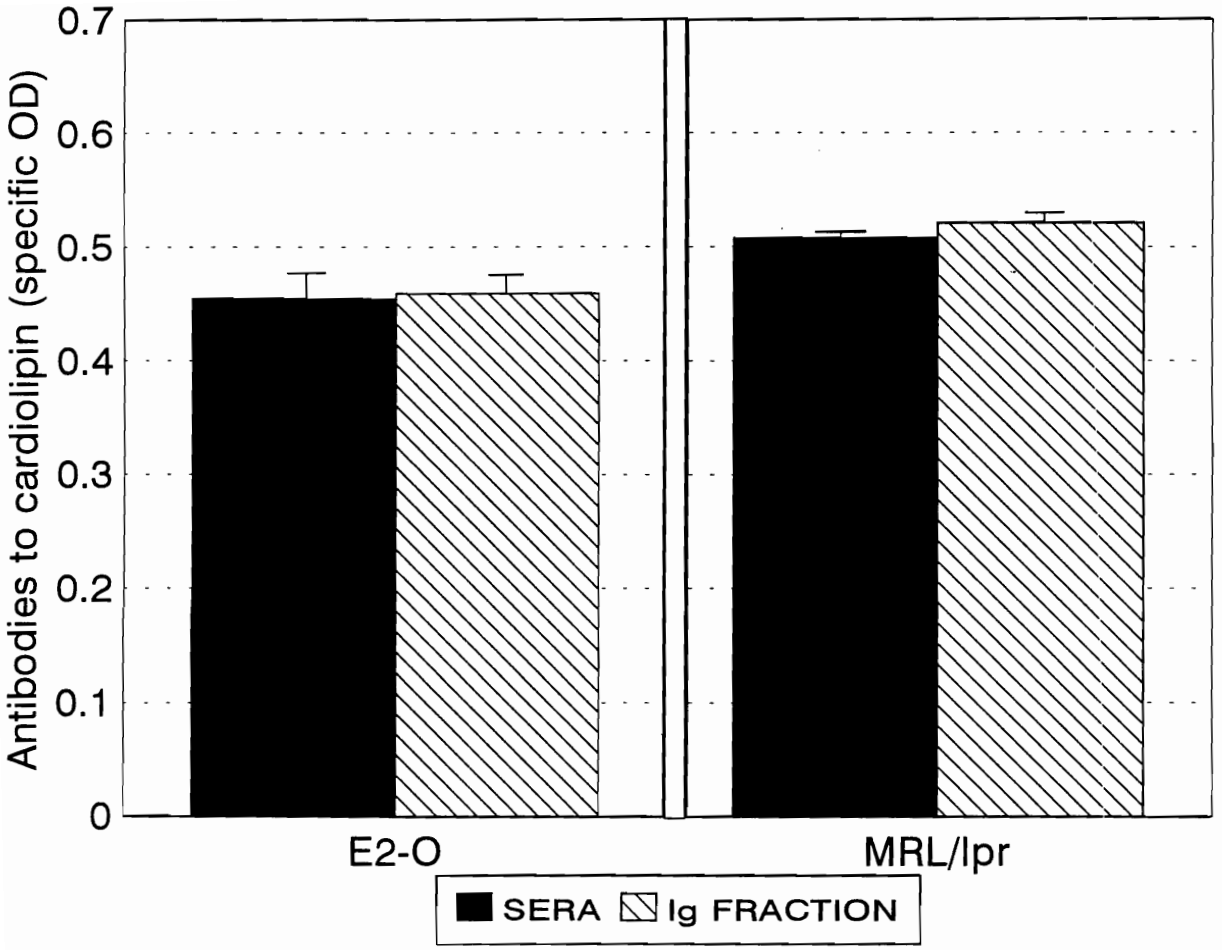


Figure 5.6: Shows that anticardiolipin antibodies bind to cardiolipin-coated plates in the absence of endogenous β2-glycoprotein I. (Note: Sera and immunoglobulin fractions from estrogen-treated C57BL/6 mice were diluted to 1:100, while the samples from MRL/lpr mice were diluted to 1:200). The binding of antibodies to cardiolipin from the immunoglobulin fraction derived from sera of C57BL/6 (n=8) and MRL/lpr mice (n=8) was similar to that of the original sera.

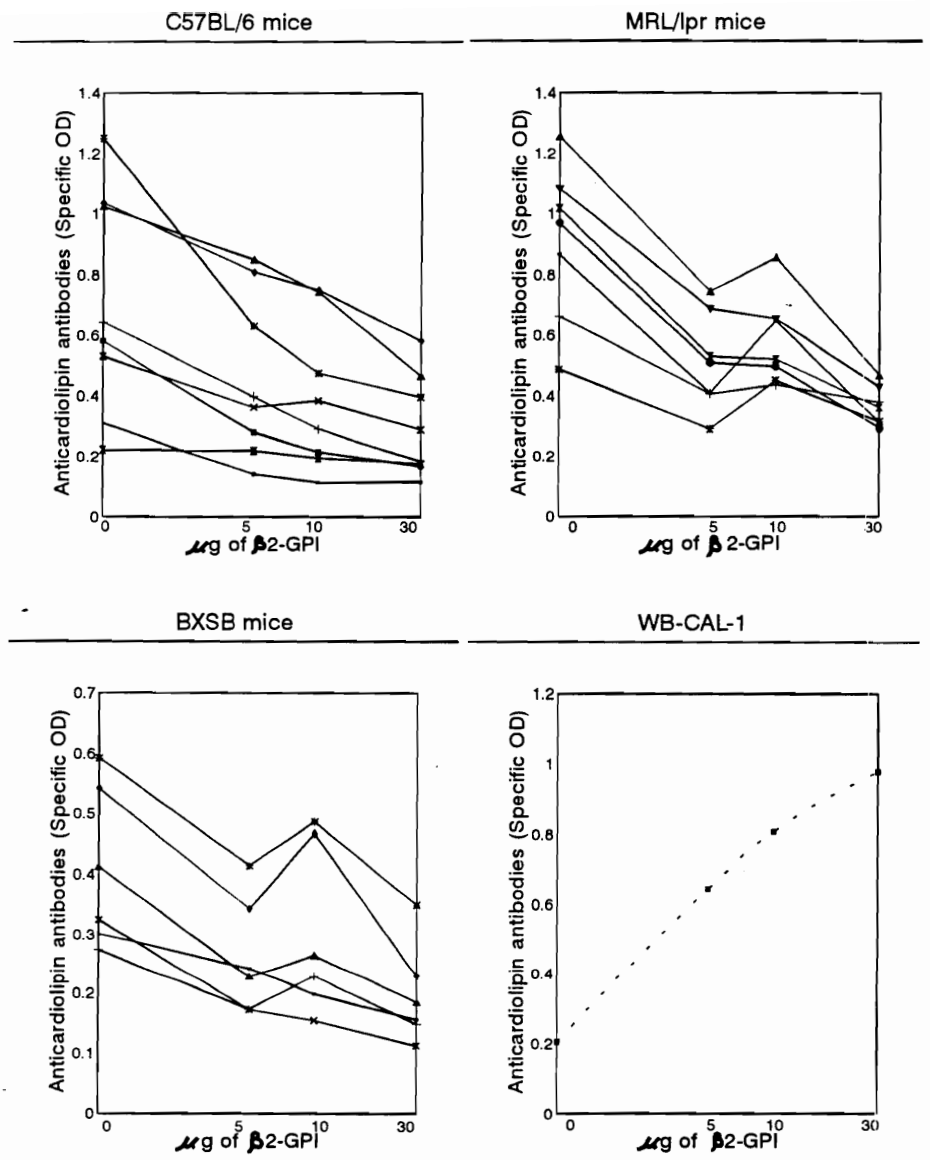


Figure 5.7: Immunoglobulin fractions from estrogen-treated C57BL/6, MRL/lpr and BXSB mice were tested for anticardiolipin antibodies in the presence of increasing concentrations of β2-glycoprotein I. This figure shows that the addition of exogenous human β2-glycoprotein I reduced rather than enhanced the binding of anticardiolipin antibodies in all three strains ($p < 0.01$). Note, that the binding of monoclonal antibody WB-CAL-1 to cardiolipin was increased with the addition of β2-glycoprotein I.

CHAPTER 6:

ESTROGEN INDUCES B CELL ACTIVATION IN NORMAL MICE: DIFFERENTIAL EFFECT IN SPLEEN AND BONE MARROW

Verthelyi D. and Ansar Ahmed S.

Estrogen induces B cell activation in normal mice: differential effect in spleen and bone marrow. To be submitteed to Cellular Immunology.

ABSTRACT:

For many years the immune system was thought to work in isolation. In recent years our understanding of the immune system has improved, and intricate interactions between the immune and endocrine systems have now been recognized. In previous studies we had shown that estrogen-treatment of nonautoimmune mice elicited a long lasting expression of IgG antibodies to dsDNA and cardiolipin. In this study we extend these serologic findings to demonstrate that estrogen induces antibodies not only to self antigens, but to foreign antigens as well. Further, the autoantibody-promoting effects of estrogen on B cells from spleen and bone marrow were studied by employing ELISPOT, image analytical and microscopic procedures. Our findings suggest that estrogen promotes the differentiation of resting B cells to plasma cells, and increases the number of antibody-forming cells as well as the antibody yield per cell. It is of particular interest that the B cell repertoire from bone marrow, but not from spleen, was shifted towards autoantibody production.

INTRODUCTION:

Studies in humans and in animal models of autoimmune diseases have clearly shown that sex steroid hormones have a profound effect on the immune system (Ansar Ahmed et al., 1985a; Lahita, 1990; daSilva, 1995). As an example, estrogen has been shown to accelerate lupus-like disease in (NZB x NZW) F1 mice (Roubinian et al.,1978). A majority of the studies to date, however, have focused on the effects of sex hormones on strains that spontaneously develop autoimmune disorders. In these mice, estrogen has been shown to modulate the course of the disease (reviewed in Ansar Ahmed and Talal, 1990)

In previous studies, utilizing C57BL/6 and BALB/c mice, we have shown that nonautoimmune mice develop autoantibodies specific for bromelin-treated erythrocytes (Ansar Ahmed et al., 1989), dsDNA (Verthelyi and Ansar Ahmed, 1994), cardiolipin and other anionic membrane phospholipids (Ansar Ahmed and Verthelyi, 1993; Verthelyi, Schurig and Ansar Ahmed, unpublished) when exposed to exogenous estrogen. Estrogen-induced autoantibodies to dsDNA and cardiolipin are long lasting. These antibodies belonged to IgM and IgG, but not IgA, isotypes, and the main subisotypes elicited are IgG2b and IgG1 (Verthelyi and Ansar Ahmed, 1994; Verthelyi and Ansar Ahmed, 1996).

In this study, we show that estrogen treatment of mice induces a variety of IgG antibodies to "self" as well as foreign antigens in sera. These increases in antibodies correlated with an increased proportion of plasma cells in the spleen. At the cellular level, using an ELISPOT assay coupled with morphometric analysis of the spots

generated, we found that estrogen induces an increase in both, the number of activated B cells, as well as the antibody yield per B cell. This increase was evident for lymphocytes derived from spleen and bone marrow. Interestingly, we found that while spleen lymphocytes had an increase in antibody forming cells for all antigenic specificities tested, active antibody forming cells from bone marrow recognized preferentially the autoantigens (cardiolipin and dsDNA).

MATERIALS AND METHODS:

Mice: Three-week old male C57BL/6J purchased from the Jackson Laboratories (Bar Harbour, Maine) were housed in standard cages (3 to 5 mice/cage) with a 14 light/10 dark hours-schedule and fed on a commercial diet. All experimental animals were cared for and maintained as per the VPI&SU institutional guidelines.

Sex hormonal treatment: Four to five-week old male C57BL/6 mice were orchiectomized as described previously (Ansar Ahmed et al., 1986; Ansar Ahmed et al., 1989). Silastic implants (Dow Corning Co., MI) containing 4-6 mg of 17β-estradiol (E2; Sigma, St. Louis, MO), were surgically placed subcutaneously as reported earlier (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994). Silastic implants (7mm long) release hormones slowly for several months (Ansar Ahmed et al., 1989). Mice with gonadectomies that received placebo implants served as controls. We had previously reported (Verthelyi and Ansar Ahmed, 1994) that the mean estrogen levels achieved by this method tripled the levels found in gonadectomized mice that only received placebos. Additional assessment of estrogen activity *in vivo* included: (1) osteopetrosis of the long bones and reduced bone marrow cellularity (Seaman et al., 1978; Ansar Ahmed et al.,

1989); (2) atrophy of the thymus and depletion of thymocytes numbers (Ansar Ahmed et al., 1985); and (3) distension of urinary bladder (Keisler et al., 1991).

Serum collection: Serum from each mouse was isolated from blood collected retroorbitally at the time of death. Sera were kept at -70°C until tested for the presence of autoantibodies.

Isolation of lymphocytes and purification of B cells: Lymphocytes from bone marrow and spleen were isolated and enumerated as described in our earlier studies (Ansar Ahmed et al., 1989). Briefly, spleens were collected under sterile conditions, gently minced and passed through a wire mesh to produce a single cell suspension. Cells were then washed twice with incomplete RPMI media. Red blood cells were eliminated by ACK lysis buffer and washed twice again. Cells were counted and resuspended in RPMI media-10 % fetal calf sera (FCS) containing non-essential aminoacids, 2mM L-glutamine and 2-mercaptoethanol (50 μg/ml). Bone marrow lymphocytes were obtained by flushing long bones (femur and humerus) with RPMI-1640 media and processed in a similar fashion. Cell numbers and viability were assessed by the trypan blue exclusion method. Lymphocytes were resuspended in complete media containing 10% fetal calf serum.

Splenic B cells were enriched by cytolytic elimination of T cells. Splenic lymphocytes were incubated in the presence of affinity-purified rat anti-mouse Thy1.2 (10 ug/10^7 cells; ATCC cell line, TIB 107, purified on a protein G column) for 45 minutes at 4°C. Cells were then washed and resuspended at 10^7 cells/ml in low-tox baby rabbit complement (1:10 in RPMI; Pel Freeze, Brown Deer, WI) for 30 minutes at room temperature. Cells were then washed three times and resuspended in fetal calf serum-

supplemented complete media. The enrichment of B cells in the resulting cell preparation was determined by staining with phycoerythrin-conjugated rat anti-mouse CD45R antibodies (1μg/10^6 cells; Pharmingen, San Diego, CA) and analyzed by flow cytometry (Epics Coulter,XL/MXL). Greater than 90 % (range of 90-97%) of the cells were positively stained with this antibody.

Quantification of plasma cells in spleen: Segments of freshly collected spleens (9 from estrogen treated mice and 9 from orchiectomized controls) were imprinted on a glass microscope slide, air dried and then stained with Wright's stain. A differential count of 1000 cells was performed under 100X magnification. Plasma cells were enumerated. Samples were read blind and decoded retrospectively.

ELISA assays: Antibodies in sera were assessed by the standard ELISA as described previously (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994). Antibodies to cardiolipin were tested on medium-binding 96-well flat bottom plates while high-binding plates (Costar, Cambridge, MA) were used for all other antigens. Plates used to assess antibodies to dsDNA were coated with methylated bovine serum albumin (BSA) for 6 hours at 4°C prior to the addition of the S1 nuclease-treated dsDNA (70 μg/ml in tris EDTA, pH 8.0). Cardiolipin (Sigma, MO) was coated at 50 μg/ml in ethanol. Other antigens used included: actin (10 μg/ml in carbonate-bicarbonate buffer) (Klinman et al.,1994), ovalbumin (1 mg/ml in PBS), lysozyme (1 μg/ml in PBS), acetone-killed *Brucella abortus* strain RB51 (2 μg/ml in carbonate bicarbonate buffer) and *Actinobacillus pleuropneumoniae* serotype 7 strain 29628 (10^9 CFU/ml in 1% formalin/PBS diluted 1:5 in carbonate bicarbonate buffer). Antigen-coated plates were incubated overnight at 4°C overnight. After washing with PBS, the wells were blocked

with PBS containing 2% bovine serum albumin (BSA) for 90 minutes. The wells were then washed three times with PBS. Serum samples (1:100 dilution in PBS 1% BSA) were incubated in the wells for 3 hours at room temperature. After washing, alkaline phosphatase-conjugated goat anti-mouse heavy-chain specific immunoglobulins (1:3000; Southern Biotechnologies Assoc. Birmingham, AL.) were added. The plates were incubated for 1 hour at 37°C in a humid chamber. The wells were washed again and then developed with p-nitrophenylphosphate (Sigma, MO) in diethanolamine buffer (pH 9.8) and read after 60 minutes (405 nm, Molecular Devices, Menlo Park, CA). Negative controls included wells with all reagents except serum ("no serum blanks"), or with no antigen ("no antigen blanks"). In addition, known positive controls for cardiolipin, dsDNA and ***Brucella abortus*** were included to monitor the validity of the assays. Absorbancies are expressed as specific OD which were obtained by: (mean OD of sample *minus* mean OD of "no serum blanks" on the plate) (Verthelyi and Ansar Ahmed, 1994).

ELISPOT assays: The number of B cells actively producing antibodies were enumerated utilizing an ELISPOT assay. The principle of the ELISPOT assay is that immunoglobulins synthesized by cells in culture bind at the site of the cell and when the wells are developed these antibodies can be identified as a spot (**Figure 6.1**). The ELISPOT assay was performed as described (Ando et al., 1986; Klinman and Steinberg, 1987) with minor modifications. Briefly, 96 well-flat bottom plates (Costar, high binding ELISA plates) were coated with dsDNA (Sigma, St Louis, MO; 70 μg/ml in PBS), cardiolipin (Sigma, St Louis, MO; 50 μg/ml in ethanol), actin (Sigma, St Louis, MO; 10 μg/ml in PBS), ovoalbumin (Sigma, St Louis, MO; 0.1%) or heavy chain specific goat anti-mouse immunoglobulin (Southern Biotechnology Assoc., Birmingham, AL., 1 μg/ml in PBS). Plates were covered and incubated for 48 hours under sterile conditions

at 4°C. Plates were then washed with PBS and blocked for 2 hours with 125 μl per well of PBS-2% BSA (pH 7.1). Dilutions of single cell suspensions (100 ul) from the spleen were the plated at 5 to 0.2 x 10^6 cells per ml for B cells producing antibodies to cardiolipin, dsDNA, actin or ovoalbumin, and 0.2 to 1 x 10^5 cells per ml for B cells actively secreting IgG or IgM immunoglobulins. Care was taken not to disturb the cells during incubation. Since estrogen induces a severe depletion of bone marrow cells, these cells were plated at 0.5 to 5 X 10^5 cells per ml for specific antigens and 0.1 to 1 x 10^5 cells per ml in wells coated with anti-IgG or anti-IgM. Cells were incubated for 6 hours at 37°C in 5% CO_2 incubators. Wells were thoroughly washed (5 times) with PBS and alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (1: 3000, Southern Biotechnology, Birmingham, AL) in PBS 2% BSA were used as secondary antibodies. Five-bromo-4-chloro-3-indolyl phosphate (BCIP) at 2.3 mM in 2-amino-2-methyl-1-propanol buffer (AMP buffer) was mixed in 4:1 ratio with 3% agarose to be used as substrate. The number of spots (each representing an antibody-producing cell) were counted under a 10X magnification. Results are shown as the number of cells actively secreting antibodies per 10^6 cells.

Morphometric analysis: Quantification of the size and density of the spots was performed utilizing a color image analyzer (Olympus Cue 3 software; Galai Production LTD. Israel) **(Figure 6.2)**. Nine to twelve wells per antigen and isotype were assessed for each treatment group. The spots image was recorded using a CCI single chip camera (Autotechnica 852). Spots were digitalized and analyzed for area and density. Spots that were too close to be analyzed independently were gated out of the analysis. The sensitivity thresholds were maintained constant for all wells. The data is presented as the mean density and area of the spots on each well.

Statistical Analysis: Non-parametric ANOVA or Mann-Whitney tests were employed given the lack of normality of the distribution of the data. Accordingly median are used to describe the data (therefore, standard errors were not included)

RESULTS:

Nonautoimmune mice treated with estrogen express antibodies to a variety of auto and heteroantigens: In previous studies we had shown that estrogen induced the expression of IgM and IgG antibodies to dsDNA, cardiolipin and other membrane phospholipids. These antigens share similar a backbone structure as well as a strong negative charge (Lafer et al.,1981). To assess whether the increase in antibodies was restricted to these negatively charged autoantigens, we tested the sera of male C57BL/6 mice that were either treated with estrogen or placebo implants with a panel of auto and heteroantigens. As shown in **Figure 6.3**, estrogen treated non-autoimmune C57BL/6 expressed increased amounts of IgG antibodies to self antigens cardiolipin, dsDNA and actin, and also to foreign proteins, such as ovoalbumin, lysozyme, and two complex microbial antigens: acetone-killed *Brucella abortus* strain RB51 and *Actinobacillus pleuropneumoniae*. Estrogen also significantly augmented the level of IgM antibodies to dsDNA, cardiolipin, lysozyme, and *B. abortus* (data not shown).

Estrogen induces an increase in the number of splenic plasma cells: An increase in serum antibodies could result from (i) an increase in the number of plasma cells, (ii) augmented production of antibodies per cell, or (iii) a combination of the above. To assess whether estrogen induced an increase in the number of plasma cells in the spleen, imprints of thin sections of spleens were fixed on microscope slides and stained with

Wright's stain **(Figure 6.4)**. Differential counts of spleen cells revealed a ten fold ($p<0.005$) higher the number of plasma cells (3.385% and 0.36%, respectively) in spleens from estrogen-treated orchiectomized mice compared to orchiectomized placebo-treated control mice.

Estrogen induces an increase in the number of spleen and bone marrow lymphocytes spontaneously secreting IgG and IgM immunoglobulins: The above observations suggested that estrogen augmented the number of immunoglobulin producing cells. To further explore the effects of estrogen on B cell function, we employed an ELISPOT assay to quantitate the number of spleen and bone marrow lymphocytes from male estrogen-treated and placebo-control mice that actively secrete immunoglobulins in the absence of stimuli. As shown in **Figure 6.5**, estrogen induced a significant increase in the number of splenic lymphocytes that spontaneously secrete IgG and IgM immunoglobulins compared to controls (10 fold and 3 fold increase, respectively; $p< 0.001$). Lymphocytes from bone marrow, however, showed a significant increase in the number of IgG actively secreting cells (13 fold increase; $p<0.001$) but no significant difference for IgM secreting cells (less than 2 fold increase; statistically not significant).

Estrogen not only induces an increase in the number of B cells actively secreting antibodies, but also appears to boost the immunoglobulin yield per cell: We had observed that the wells that had lymphocytes from estrogen-treated mice not only had more spots, but also that the spots appeared to be larger and more intensely stained. To quantify the size and density of the spots in individual wells, we utilized a color image analyzer (Galai Productions Ltd., Israel). As shown in **figure 6.6**, individual spots made

by spleen and bone marrow lymphocytes of IgG isotype from estrogen-treated mice are significantly larger and denser than those of placebo-controls. These results indicate that estrogen not only induces an increase in the number of activated B cells, but also augments the amount of immunoglobulin secreted by individual cells.

Similar to what was observed with regards to the number of antibody producing cells, the differences in area and density of the spots made by splenic lymphocytes from estrogen- and placebo- treated mice were less remarkable for IgM splenic lymphocytes, and not significant for IgM lymphocytes from bone marrow.

Estrogen increases the number of lymphocytes from spleen that produce antibodies to auto and heteroantigens: Increased B cell activity present in autoimmune diseases has been has been attributed to a generalized increase in antibody-producing cells or to the preferential expansion of autoreactive clones (Reviewed in Theofiloupolus, 1995).

Spleen cells from mice treated with placebos or estrogen implants were utilized to assess whether estrogen elicited a generalized increase in activated antibody forming cells or a selective increase in the number of B cells spontaneously secreting autoantibodies. The number of cells actively producing antibodies to cardiolipin and dsDNA (self antibodies frequently associated with autoimmune diseases), actin (self antigen not usually associated with systemic autoimmune diseases), and ovalbumin (heteroantigen) were quantitated by ELISPOT. As shown in **figure 6.7a,** orchiectomized nonautoimmune C57BL/6 mice treated with placebos had nearly no detectable antibody-forming splenic cells that spontaneously produced IgG immunoglobulins to the antigens tested. Splenic lymphocytes from estrogen-treated mice showed a significant increase in the expression

of cells spontaneously secreting IgG antibodies to self antigens cardiolipin, dsDNA and actin, but not to ovoalbumin.

Placebo-treated mice showed low levels of active antibody forming cells of IgM isotype to cardiolipin, actin, dsDNA and ovoalbumin. Estrogen induced a significant increase in the number of antibody forming cells for all specificities tested **(Figure 6.7b)**.

To assess whether the increase in antibody forming cells reflected a shift in the spectra of cells spontaneously secreting antibodies in estrogen treated mice, we simultaneously quantitated both, the total and the antigen-directed immunoglobulin-secreting cells of IgG and IgM isotype. It is not possible to directly determine the absolute number of B cells secreting specific antibodies from the total number of immunoglobulin-secreting B cells because different cell dilutions and various reagents of differing degrees of sensitivity were employed. Nevertheless, a comparison between groups, estrogen-treated *versus* control mice, could be made. Analyzing the data in this manner **(figure 6.7 e & f)** allowed us to determine that mice from both treatment groups (E2-O and P-O) devoted similar proportions of B cells to each antigen. These results suggest that estrogen may act on splenic lymphocytes as a polyclonal B cell activator, and the resulting increase in autoantibodies is a manifestation of the overall increase in immunoglobulin-producing-cells.

Bone marrow: Mature antibody-forming cells have been shown to relocate to the bone marrow (Benner et al., 1981). Therefore, bone marrow cells from orchiectomized male mice treated with estrogen or placebo were tested for the presence of active antibody-forming cells to cardiolipin, dsDNA, actin and ovoalbumin. The number of activated

antibody-forming cells that bound to these antigens in the placebo-treated orchiectomized mice was very low. **Figure 6.7 c & d** shows that estrogen induced a significant increase in the number of immunoglobulin-secreting cells (both IgG and IgM) to all antigens tested, but mainly to cardiolipin and dsDNA.

The number of spots elicited for individual antigens were then compared to the total number of immunoglobulin-secreting cells of the same isotype for each individual mouse. Bone marrow lymphocytes **(figure 6.7g & h)**, in contrast to those from spleen, showed a significant preferential activation of IgM and IgG antibody-secreting cells directed to cardiolipin ($p < 0.001$) and dsDNA ($p < 0.01$).

Antibody yield per cell in spleen and bone marrow: The area and density of the spots generated by the ELISPOT assay were determined to assess whether estrogen preferentially enhanced the immunoglobulin yield of autoantibody producing cells. The mean area and density of the spots in nine to twelve wells were assessed for each antigen. Although IgG immunoglobulin producing cells of spleen and bone marrow from estrogen-treated mice had shown very significant increase in area and density compared to the placebo-treated controls, the minimal number of cells spontaneously secreting IgG antibodies specific for the antigens tested in placebo-treated mice precluded morphometric comparison of the spots.

Morphometric characterization of bone marrow spots elicited by lymphocytes of IgM isotype indicated a significant increase in the area and density of the spots of anticardiolipin-forming cells from estrogen-treated mice. Spots formed by bone marrow of other specificities although larger and denser, were not statistically different from

those made by the controls **(figure 6.8 top panels)**. In spleen, IgM lymphocytes from estrogen-treated mice showed a tendency towards increased area and density, but differences were not significant (**figure 6.8 bottom panels**).

Purified splenic B cells from estrogen-treated mice show increased number of activated immunoglobulin/antibody-secreting cells of IgG and IgM isotype: To assess whether the estrogen-induced increase in the number of cells spontaneously secreting immunoglobulins was dependent upon the presence of T cells and macrophages in culture, we performed the ELISPOT assays utilizing purified splenic B cells from placebo- and estrogen-treated mice. The reduced number of cells available from bone marrow in mice treated with estrogen precluded the determination of antibody forming cells in purified bone marrow B cells. In spleen, lymphocytes from estrogen-treated mice had increased numbers of cells actively secreting immunoglobulins of both IgG and IgM isotype ($p < 0.05$). Purified B cells from placebo-treated mice had no detectable IgG antibody-producing cells (and only a few of the IgM isotype) specific for cardiolipin, actin or ovoalbumin (data not shown). B lymphocytes from estrogen-treated mice were positive for IgG antibody-producing cells to cardiolipin, actin and ovoalbumin, and showed a 10 fold increase in the number of activated IgM B cells specific for these antigens (data not shown).

DISCUSSION:

Sex hormones have been found to modulate the immune system (Ansar Ahmed et al., 1985; Schuurs and Verheul, 1990; da Silva, 1995). Administration of estrogen to mice genetically-prone to autoimmune disease ((NZB x NZW)F_1 and MRL/lpr), accelerated

the development of autoantibodies, lymphadenopathy, arthritis, immune complex glomerulonephritis and death (Roubinian et al., 1978; Siiteri et al., 1979; Carlsten et al, 1990; Carlsten et al., 1991). Similarly, in non-spontaneous models for SLE such as BALB/c mice injected with anti-DNA (16/6 id+) antibodies, estrogen treatment accelerated the development of autoantibodies (Blank et al., 1990). Antibodies reactive to self-antigens are present in normal individuals as part of their natural antibody repertoire. These antibodies are usually broadly crossreactive, have low affinity and generally are of the IgM isotype (Avrameas, 1986; Schwartz, 1993). Natural autoantibodies are thought to be important in protecting the host from pathogens before a more specific immune response is in place, as well as in the elimination of degraded self antigens (reviewed in Avrameas, 1986; Schwartz, 1993). The expression of these autoantibodies, *in vivo,* is tightly regulated to prevent the potential development of high affinity autoantibodies and autoimmune disease. Autoantibodies that are present in autoimmune diseases, such as SLE, RA and SS are, however, usually of high affinity, oligospecific and mainly of the IgG isotype. Although the precise pathogenic role of these autoantibodies has remained elusive (Hahn and Tsao, 1993; Emlen et al., 1986), the pathogenic importance of B cells and the autoantibodies have been recently underscored by studies on Jh knockout mice (which are unable to produce mature B cells) with lpr/lpr background. These mice showed no signs of kidney or vascular lesions (Shlomchick et al., 1994).

In nonautoimmune mice, estrogen has been shown to accelerate the expression of total or specific antibodies in C3H, C57BL/6J, but not in DBA/1 or NFR/N mice (Carlsten et al., 1989; Nilsson and Carlsten, 1994; Ansar Ahmed et al., 1989; Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994). We have recently found that

estrogen-treated BALB/c mice also have increased levels of antibodies to dsDNA, cardiolipin and *B. abortus* (Verthelyi, Ansar Ahmed, and Schurig, unpublished). In this study we show that nonautoimmune C57BL/6 mice when treated with estrogen develop increased levels of antibodies to a variety of auto- and hetero-antigens. These results suggests that estrogen-induced B cell activation is not restricted to clones that target negatively charged autoantigens, but rather encompasses a wide variety of specificities. Importantly, inhibition studies showed that the crossreactivity among these estrogen-induced anticardiolipin antibodies with heteroantibodies was very low (Verthelyi and Ansar Ahmed, 1996).

At the cellular level, we have shown that estrogen-treated mice have increased numbers of plasma cells in the spleen, and that these plasma cells actively secrete immunoglobulins of IgM as well as IgG isotype. As assessed by ELISPOT assay, splenic lymphocytes from estrogen-treated C57BL/6 mice showed a 10 fold increase in IgG producing cells and a 3 fold increase in IgM producing cells. These results resemble those obtained by treating autoimmune-prone MRL/lpr mice treated with estrogen (Nilsson and Carlsten, 1994; Brick et al., 1985). In nonautoimmune mice treated with estrogen splenic B cells not only had increased number of antibody-forming cells, but the antibody yield per cell was higher as well. Our results concur only partially with previous studies in Sprague Dawley rats, in which estrogen was shown to induce higher IgM antibody titer, but no increase in the number of the number of plaque forming cells (Myers and Petersen, 1985). It is important to point out that, as reported by others (Brick et al,., 1985), the estrogen-mediated increase in immunoglobulin forming cells was not a manifestation of increased numbers of B cells in spleen. Quantification of $CD45R^+$ cells by flow cytometry determined that the number of splenic B cells in mice

that had received estrogen were either equal or reduced compared to the placebo-treated controls (Verthelyi and Ansar Ahmed unpublished).

Several mechanisms have been postulated for the increase in B cell activity and expression of autoantibodies in autoimmune mice. Some investigators postulate that the diversity of autoantibodies is due to generalized B cell activation (polyclonal B cell activation) which is independent of their specificity; this would include clones that are under normal conditions tightly regulated (Theofilopoulos and Dixon, 1981; Klinman and Steinberg, 1987; Theofilopoulos, 1995). Downregulation of NK cell or suppressor T cell functions, or upregulation of Th2 cells that would allow the expansion of clones that are normally suppressed are among the potential mechanisms cited (Paavonen et al., 1981; Seaman et al., 1978; Ansar Ahmed et al., 1989b; Llorente et al.,1994). Others, think that autoreactive clones are preferentially expanded, while those targeted to conventional antigens remain quiescent (Hayakawa et al., 1984). Further, since the autoantibodies in autoimmune diseases are of IgG isotype, high affinity, and show signs of somatic mutations compatible with antigen driven response, the increase in autoantibodies could be due to an increase in the availability of self antigens (or crossreactive heteroantigens) to the immune system (Emlen et al., 1994; Theofilopoulos, 1995).

In our model, we determined that estrogen elicited in spleen the expression of active antibody forming cells to self-antigens (cardiolipin, dsDNA, actin) of IgG isotype which were not evident in mice that received placebos. Estrogen-treated mice also had an increase in the number and activity of antibody-forming cells of IgM isotype for all specificities tested. Similar increases in activated specific antibody-producing cells were

reported in autoimmune-prone (NZB x NZW)F1 mice but not DBA/2 mice (Brick et al., 1985). In our mice, however, estrogen did not appear to modify the proportion of activated antibody forming B cells that react with each antigen. This suggests that in the spleen, the increase in autoantibody producing cells reflects an overall increase in the number of active antibody-forming cells. The increase in autoantibody producing cells in splenic lymphocytes was maintained regardless of the presence or absence in culture of T cells and macrophages. This shows that the estrogen induced increase in activated immunoglobulin-producing cells is not dependent on the presence of T cells *in vitro*. This is important since increased T cell help, such as the excess production of IL-10 has been postulated as one of the mechanisms of B cell hyperactivity in SLE patients (Llorente et al., 1994).

Interestingly the effects of estrogen on bone marrow and spleen lymphocytes differ: In bone marrow, our studies show that estrogen induced an increase in IgG but not IgM immunoglobulin forming cells. Importantly, contrary to what we had observed for the spleen, bone marrow lymphocytes had a preferential expansion of autoantibodies of IgM and IgG isotype, mainly those directed against cardiolipin. This preferential stimulation of cells producing antibodies to cardiolipin is also apparent in the increase in the size and density of the spot generated, suggesting that these cells have a higher antibody per cell yield than those directed to other antigens tested. Unfortunately, the estrogen-induced reduction in the bone marrow cellularity limited the performance of studies on bone marrow lymphocytes. Bone marrow is considered to be a major source of serum immunoglobulins of IgM, IgG and IgA isotypes (reviewed in Benner et al., 1981). Early in life, bone marrow contains predominately immature B cells. Although B cells continue to be produced in bone marrow throughout life, with age, increasing numbers

of antibody-forming cells have been shown to accumulate in bone marrow (reviewed in Benner et al., 1981; Nuñez et al., 1996). Further, aged mice have increased expression of autoantibodies, and the B cell repertoire resembles that of autoimmune mice (Klinman, 1992). In aged mice, as in estrogen treated mice, there is a decrease in cellularity of the bone marrow. It is possible that altered bone marrow milieu could lead to B cell hyperactivity or a defect in the elimination of activated plasma cells due to, for example, altered apoptotic signals. This, however, would not explain the preferential increase in anticardiolipin forming cells. Alternatively, estrogen could induce a shift in lymphocyte trafficking favoring the homing of anticardiolipin forming cells to the bone marrow. A third possibility, is that estrogen-induced cell death at the bone marrow level leads to increased exposure to intracellular antigens such as cardiolipin, which may lead to expansion of these clones. These possibilities need to be further explored.

These data together with our previous findings suggest that exposure to estrogen leads to B cell hyperactivity. Further, estrogen-induced B cell-activation appears to, at least in bone marrow, disrupt the B cell repertoire to favor the expression of autoantibodies.

Acknowledgements: This study was supported by a grant from USDA, HATCH program (1-32582), NIH BSRG program (4-35032), Sigma Xi, Gina Finzi Fellowship from the Lupus Foundation of America. We thank Dr. Chickering for his expert performance of the differential counts of splenic leukocytes, and Mrs. Wolf for her invaluable help with the isolation and purification of lymphocytes.

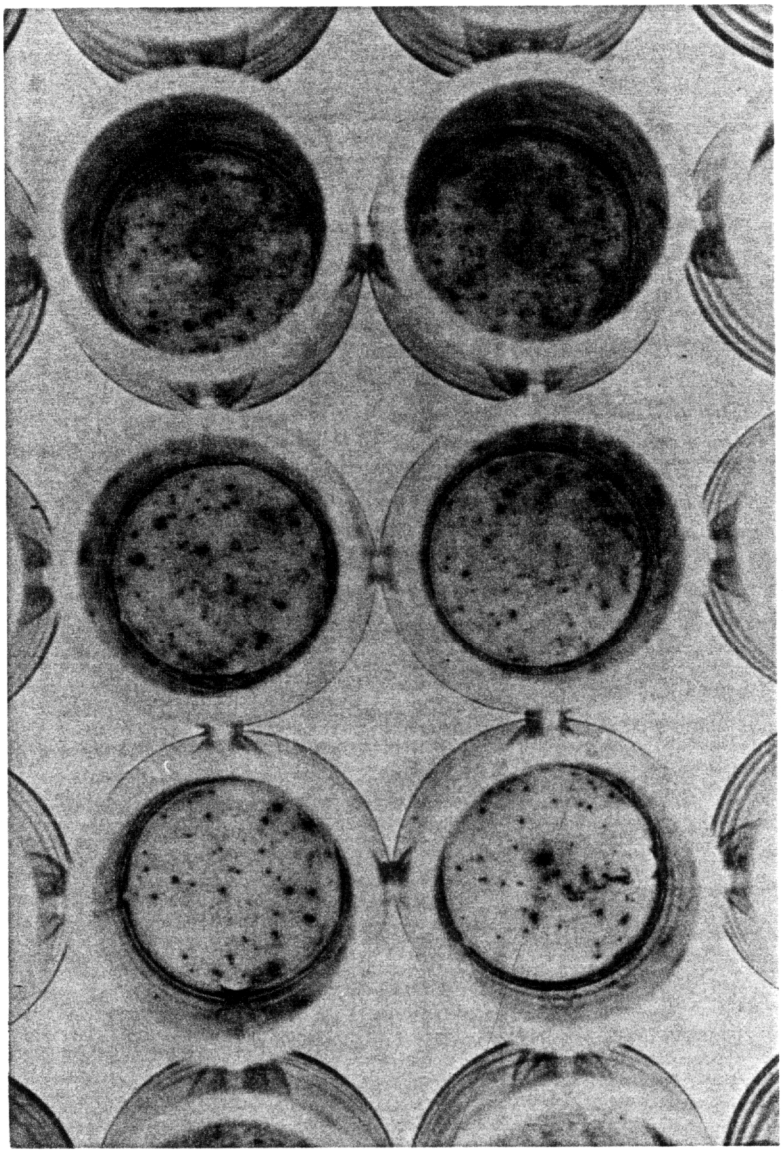


Figure 6.1: Illustrates the image generated by the ELISPOT assay.

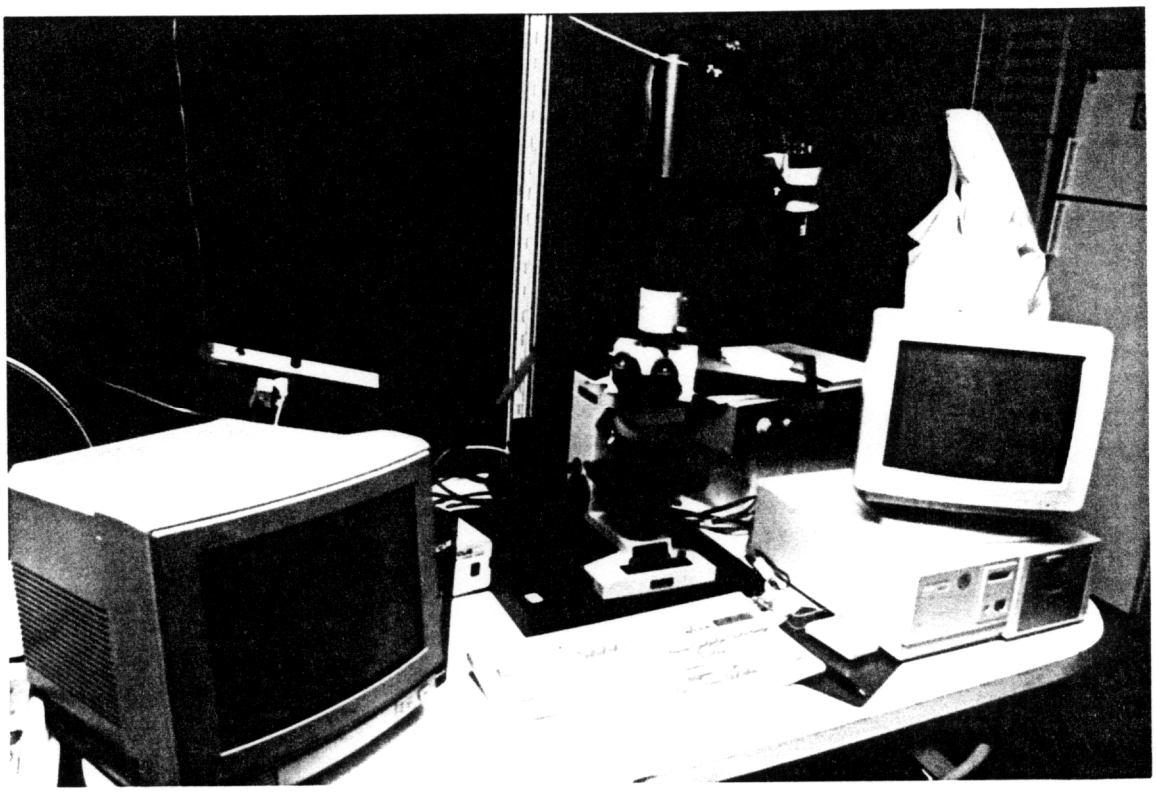


Figure 6.2: Color image analyzer used to assess the area and density of the spots generated on the ELISPOT assay.

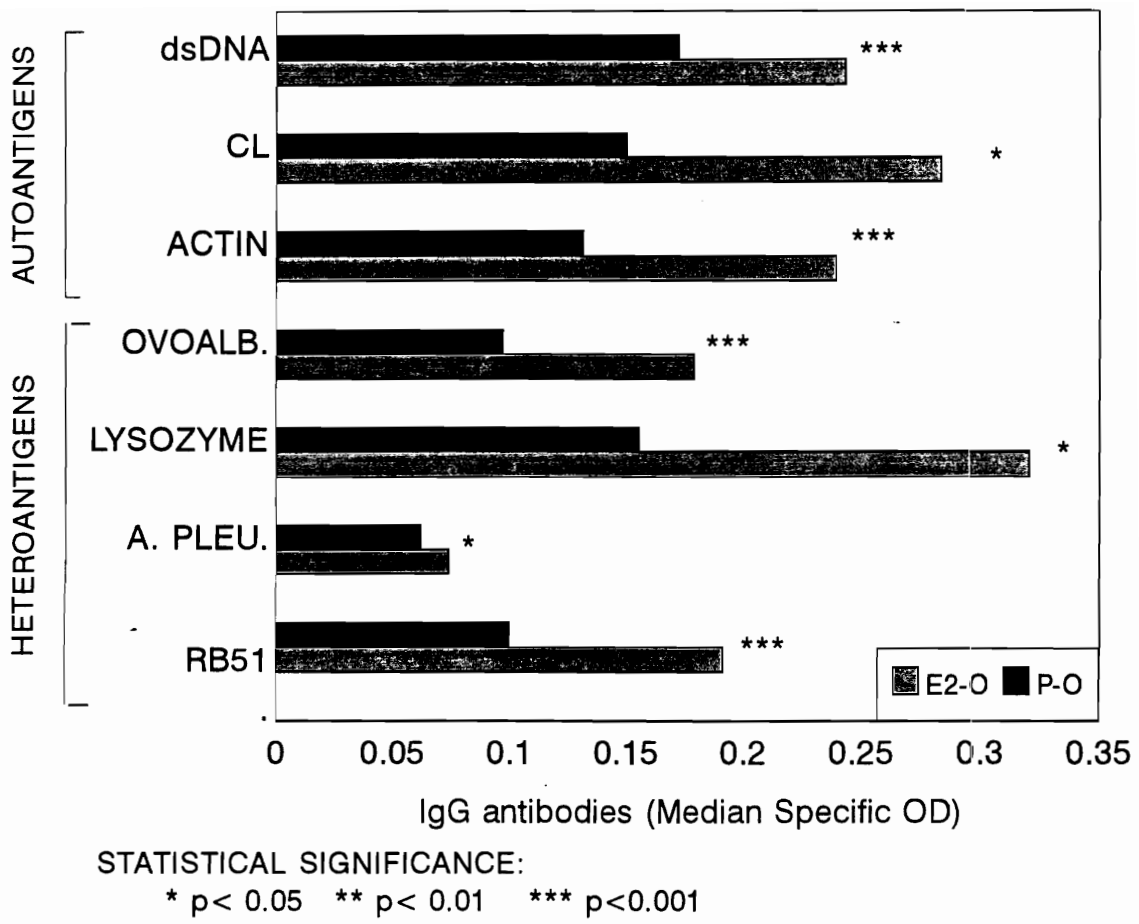


Figure 6.3: Sera from male C57BL/6 mice treated with estrogen or placebo subcutaneous implants were tested for the expression of antibodies to dsDNA, cardiolipin (CL), actin, ovalbumin (OVOALB.), heat-killed *A. pneumoniae* (ACTINOB.) and acetone-killed *B.abortus*. Since the absorbencies did not have a normal distribution, non-parametric Mann Whitney analysis was used to test for statistical differences among groups. Accordingly, the graph shows the median value for each group.

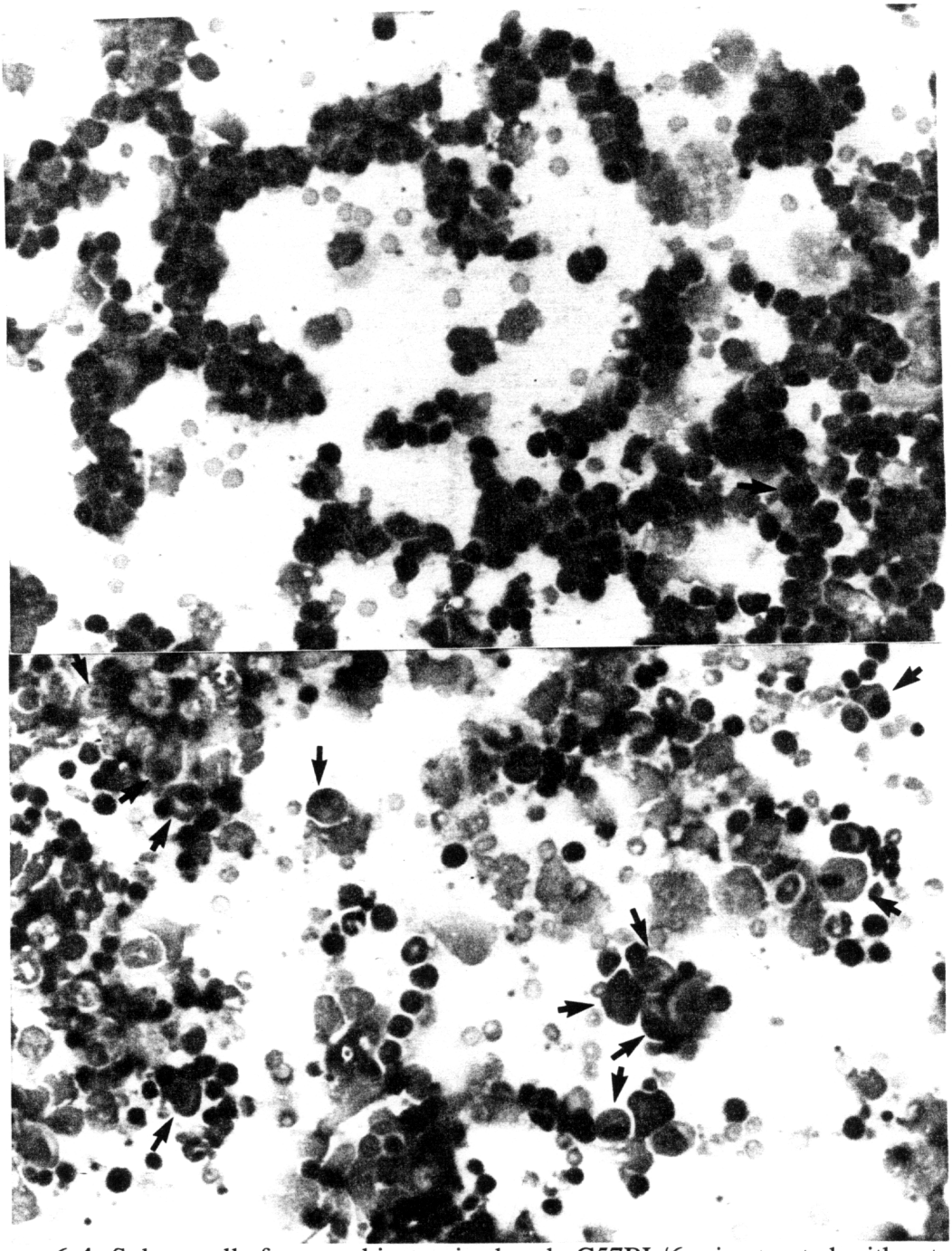


Figure 6.4: Spleen cells from orchiectomized male C57BL/6 mice treated with estrogen or placebo implants were fixed and stained. Photographs were taken under a 200X magnification. Note the increase in plasma cells in the spleen of the mouse treated with estrogen.

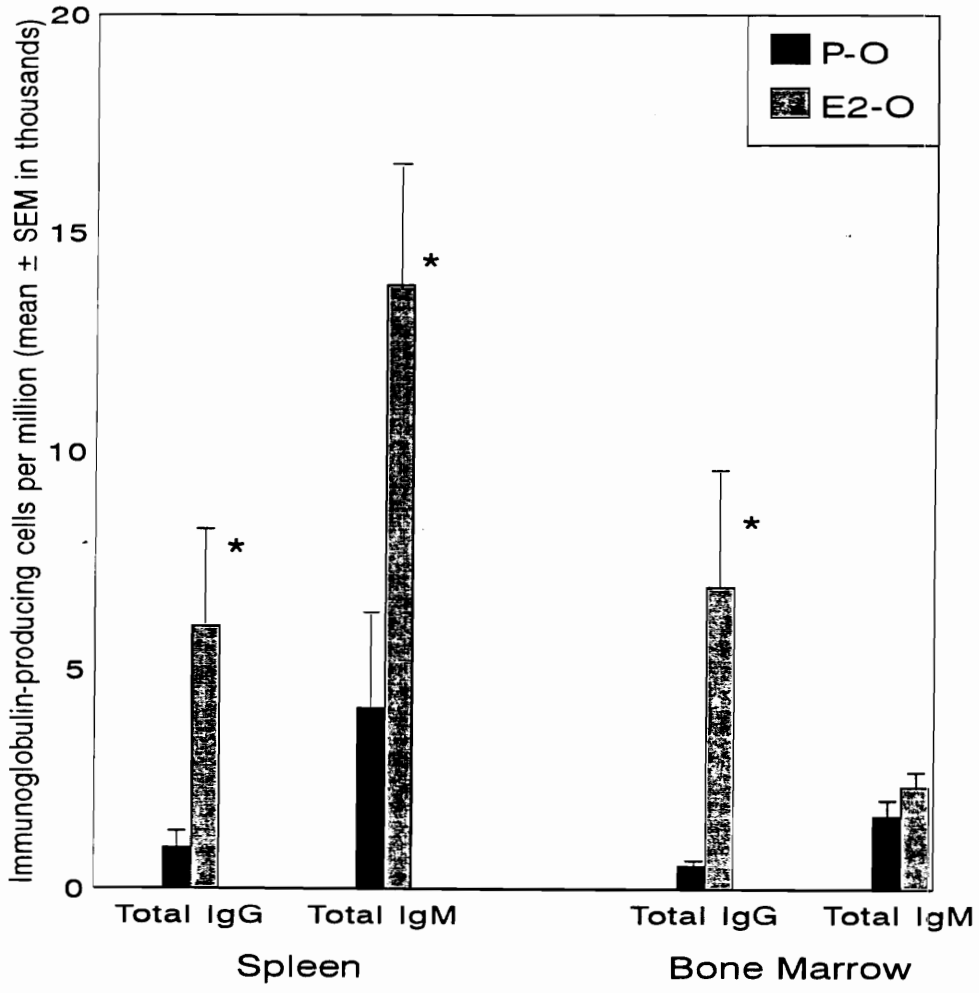


Figure 6.5: Estrogen increases the number of immunoglobulin-forming cells in spleen and bone marrow: The number of IgG and IgM immunoglobulin-forming cells per million lymphocytes derived from spleen (n=14) and bone marrow (n=12) was determined by ELISPOT. Non-parametric Mann Whitney test was used to analyze the data.

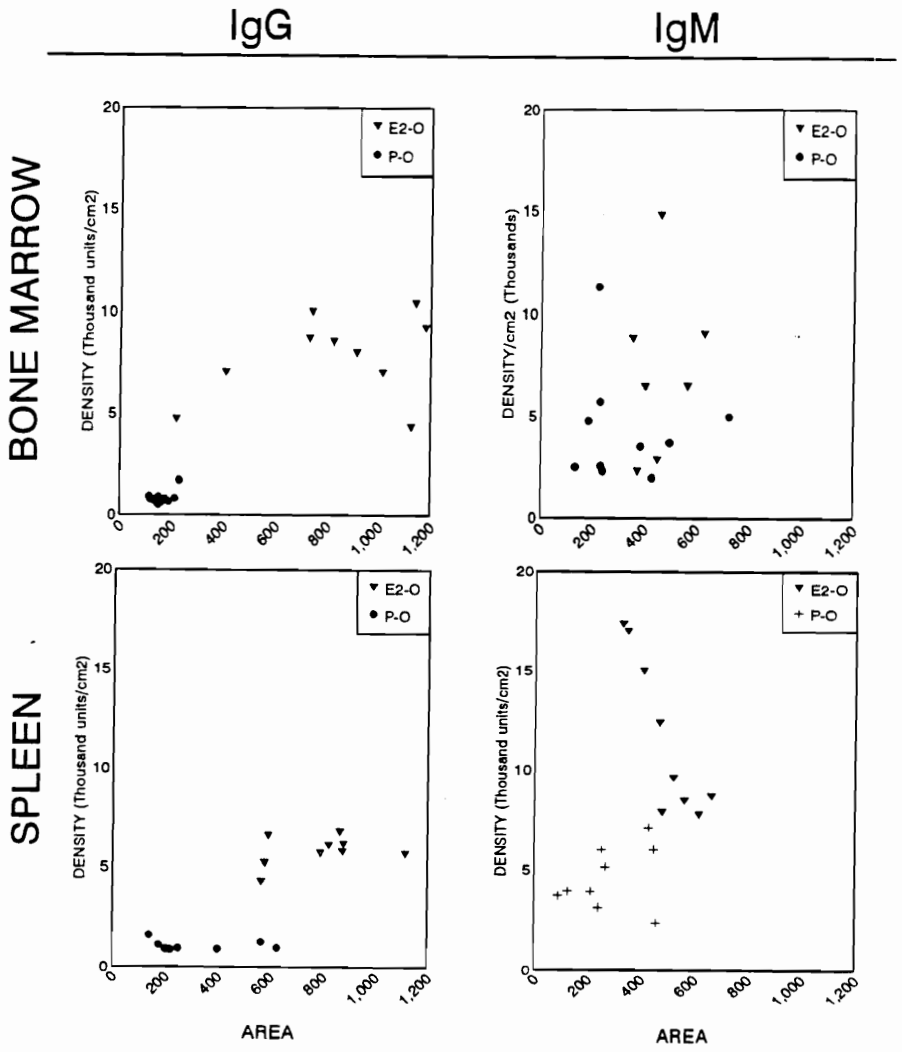


Figure 6.6: Estrogen increases the immunoglobulin yield per cell: Morphometric analysis of the area and density of the spots elicited by spleen and bone marrow lymphocytes in an ELISPOT assay was performed. Figure 6.6 illustrates the mean area and density of the spots on a well. Nine to twelve wells were assessed for each group. Non-parametric Mann whitney test was used to test differences among treatment groups.

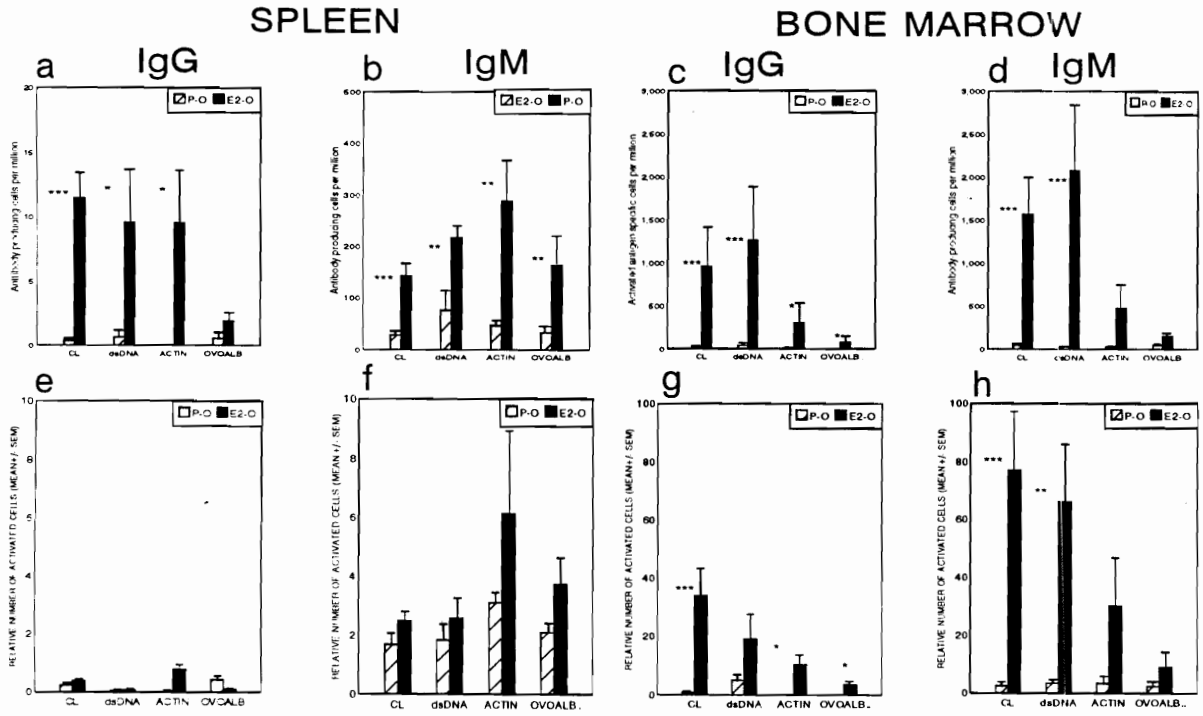
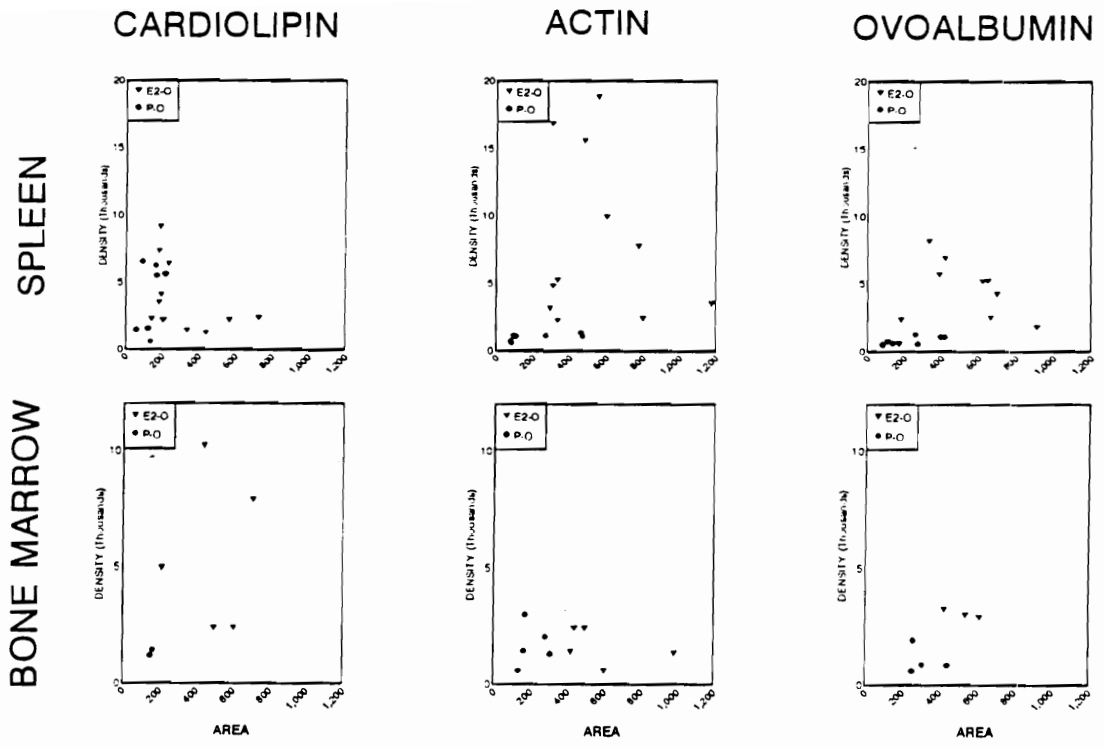


Figure 6.7: Antibody-producing cells in spleen and bone marrow: The number of antigen-specific antibody-producing cells per million lymphocytes of IgG and IgM isotype in spleen (a & b) as well as in bone marrow (c & d) are shown. Figures e-h show the number of cells actively producing antibodies that to bind a specific antigen compared to the total number of immunoglobulin-producing cells from the same organ (figure 6.5). Note that different scales were used for each graph.



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CHAPTER 7

EFFECT OF ESTROGEN ON SPLENIC B LYMPHOCYTES FROM NONAUTOIMMUNE C57BL/6 MICE: APOPTOSIS, PROLIFERATION AND CELL CYCLE

Verthelyi D and Ansar Ahmed S. Estrogen alters the proliferative and apoptosis abilities of B cells. *(Manuscript in preparation, to be submitted to J. Immunol.)*

ABSTRACT:

Sex hormones have been shown to modulate the immune system. Our previous studies had shown that exposure of nonautoimmune mice to estrogen lead to B cell hyperactivity and increased expression of autoantibodies. In these studies we have focused on the functional analysis of B cells from mice treated with estrogen. We found that while the spleen lymphocyte population from mice treated with placebos is mainly composed of small resting mature lymphocytes, spleen cells derived from mice exposed to estrogen are morphologically heterogenous. Splenic lymphocytes from estrogen-treated mice had increased numbers of spleen cells *in vivo* that were dying by apoptosis as well as an increased proportion of lymphocytes that were actively proliferating as assessed by cell cycle analysis. *In vitro*, in the absence of stimulation, B lymphocytes from mice treated with estrogen underwent active proliferation and resisted death by apoptosis, significantly more than the controls. We also found that despite their autoproliferative character, spleen B cells were able to respond adequately to stimulation with mitogens. B cells from mice treated with estrogen had a marked reduction in their susceptibility to apoptosis when in culture with LPS or antibodies to CD40 (alone or with IL-4).

Together these studies indicate that normal mice exposed to estrogen have defects in susceptibility of B cells to apoptosis as well as the ability to proliferate in the absence of stimulation. It is hoped that these studies would enhance our understanding of the immunomodulatory role of estrogen in health and in a wide range of disorders such as autoimmune and cancer disorders.

INTRODUCTION:

Studies in humans and in animal models of autoimmune diseases have clearly shown that sex steroid hormones have a profound effect on the immune system (Ansar Ahmed et al., 1985a; Lahita, 1990; daSilva, 1995). Our previous findings showed that estrogen treatment of normal C57BL/6 mice augments B cell functions (Chapters 3,4,5,& 6). This was evidenced by increased autoantibodies, increased antibodies to heteroantigens, increased number of plasma cells, increased antibody-forming cells and increased yield of antibodies per cell. Reports from other laboratories had shown that estrogen treatment induces an increased production of heteroantibodies in mice and humans (reviewed in Ansar Ahmed and Talal, 1990; Schuurs and Verheul, 1990; daSilva, 1995). However, little was known of the underlying mechanisms by which estrogen mediates these changes. We hypothesized that one of the mechanisms by which estrogen treatment leads to B cell hyperactivity and expression of autoantibodies, is by modifying the susceptibility of B cells to die by apoptosis. Apoptosis is an active form of cell death that can be readily distinguished from necrosis by several morphologic and biochemical features, including cell shrinkage, chromatin condensation and zeiosis (untethering of the cell membrane from the cytoskeleton), with relative conservation of the plasma membrane. Early on in apoptosis, the morphology of the cytoplasmic organelles is conserved, although the functional activity of the mitochondria is soon reduced (reviewed in Williams, 1995). At later stages the membrane blebs out resulting in formation of apoptotic bodies that, *in vivo,* are then phagocytized without-unlike necrosis- provoking inflammation (reviewed in Cohen, 1993; Schwartz and Osborne, 1993).

Apoptosis has been shown to play an important role in the regulation of the immune

system. Positive and negative selection of B cells as well as in the selection of memory cells involves apoptosis. Also, apoptosis of activated cells has been shown to be an important mechanism in downregulating the immune response after the antigen has been eliminated. Defects in apoptosis have been shown to lead to the expansion of autoreactive lymphocytes (reviewed in Mountz et al., 1994; Rose et al.,1994; Ogawa et al., 1995) and several animal models for autoimmune diseases show defects in apoptosis-related genes (reviewed in Rose et al., 1994). For example, in mice that spontaneously develop systemic lupus erythematosus such as MRL-lpr/lpr mice, there is a defect in the expression of the fas gene (Watanabe et al., 1992), and 'gld' mice have been shown to have a point mutation in the fas ligand gene (Watanabe,).

Estrogen has been shown to increase apoptosis of murine uterine epithelial cells (Jo et al., 1993). At sites of lymphocyte development and maturation (bone marrow and thymus), estrogen has been shown to induce cell death, but is not clear whether it is mediated by apoptosis (Smithson et al., 1995; Ansar Ahmed et al., 1985b).

To investigate the effects of estrogen on lymphocytes of nonautoimmune mice, we have first characterized the spleen cell population *ex vivo,* and then characterized the *in vitro* proliferative response and the susceptibility to apoptosis of resting and activated lymphocytes from estrogen-treated and control mice. Lipopolysaccharide and anti-CD40 antibodies were used to stimulate the splenic lymphocytes. Lipopolysaccharide has been shown to induce B cell proliferation and differentiation, block growth inhibition and apoptosis of B lymphoma cells treated with anti-immunoglobulin (reviewed in Tsubata, 1994), partially reverse clonal anergy of B cells in autoantibody-transgenic mice (reviewed in Tsubata, 1994), and prevent T cell death driven by superantigens (Vella et

al., 1995). Antibodies to CD40 have been shown to rescue B cells from apoptosis at the germinal centers, and to induce B cell proliferation and/or differentiation in resting B cells (Durie et al., 1994).

In these studies, we examined the effects of estrogen on the susceptibility to apoptosis *ex vivo*, and assess the response *in vitro* in the absence or presence of B cell stimulants the ability of B cells to proliferate and their susceptibility and kinetics of apoptosis.

MATERIALS AND METHODS:

Mice: C57BL/6J were purchased from the Jackson Laboratories (Bar Harbor, Maine). Mice were fed on a commercial diet, and housed in standard cages (3 to 5 mice/cage) with a 14 light/10 dark hours schedule as reported earlier (Verthelyi and Ansar Ahmed, 1994).

Sex hormonal treatment: Four to five-week old male C57BL/6 mice were orchiectomized as described previously (Ansar Ahmed et al., 1986; Ansar Ahmed et al., 1989). Silastic implants (Dow Corning Co., MI) containing approximately 4-6 mg of 17β-estradiol (E2; Sigma, St. Louis, MO), were surgically placed subcutaneously as reported earlier (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994). Mice with gonadectomies that received placebo implants served as controls. Assessment of estrogen activity *in vivo* included: (1) osteopetrosis of the long bones and reduced bone marrow cellularity (Seaman et al., 1978; Ansar Ahmed et al., 1989); (2) atrophy of the thymus and depletion of thymocytes numbers (Ansar Ahmed et al., 1985); (3) distension of urinary bladder (Keisler et al., 1991). In our mice, however, no blood,

proteins or other signs of infection were readily evident in urine.

Isolation of lymphocytes and purification of B cells: Splenic lymphocytes were isolated and enumerated as described in our earlier studies (Ansar Ahmed et al., 1989, please see chapter 6). Lymphocytes were resuspended in complete media (RPMI-10 % fetal calf sera (FCS) containing non essential aminoacids, 2mM L-glutamine and 2-mercaptoethanol (50 μg/ml)).

Splenic B cells were enriched by cytolytic elimination of T cells using affinity-purified rat anti-mouse Thy1.2 (10 ug/10^7 cells; ATCC cell line, TIB 107, purified on a protein G column) for 45 minutes at 4°C. Cells were then washed and resuspended at 10^7 cells/ml in low-tox baby rabbit complement (1:10 in plain RPMI; Pel Freez, Brown Deer, WI) for 30 minutes at room temperature. Cells were then washed 3 times and resuspended in complete media. The enrichment of B cells in the resulting cell preparations were determined by staining with phycoerythrin-conjugated rat anti-mouse CD45R antibodies (1μg/10^6 cells; Pharmingen, San Diego, CA) and analyzed by flow cytometry (Epics Coulter,XL/MXL). Greater than 90 % of the cells (90-97%) were positively stained with this antibody.

Proliferation assays: Lymphocytes utilized for proliferation assays were plated in a minimum of triplicates, at 5 x 10^6 cells/ ml in RPMI media containing 10 % FCS and 2mM L-glutamine. Stimulants included: LPS (10 μg/ml Sigma St Louis, MO.), anti-CD40 antibodies (5 μg/ml; a kind gift from Dr Maureen Howard from the DNAX Research Institute, Los Angeles, CA), and IL-4 (10 to 100 units/ml). One hundred μl of the appropriate dilution of the stimulants were placed in round-bottom plates (Corning,

NY) to which 100 μl of cells were added. Cells were kept in culture for 72 hours in a humidified atmosphere (5% CO_2) at 37°C. Proliferation was assessed by a nonradioactive Alamar Blue assay, as previously described (Ansar Ahmed et al., 1994). The pattern of proliferation determined by this assay was found to be similar to ^{3}H-thymidine incorporation assay (Ansar Ahmed et al.,1994). After 48 hours in culture, 20 μl of the Alamar Blue dye (Alamar, Sacramento, CA) was added to the cells. Alamar Blue contains an oxidation-reduction indicator. Proliferating cells reduce the media in the well and induce a change in color from blue to red that is proportional to the extent of the proliferation. The change in color was assessed at 72 hours by reading the absorbance at 600 nm (reduction) and 570 nm (oxidation). Proliferation was expressed as the OD at 570 nm *minus* the OD at 600 nm. Proliferation as assessed by ^{3}H-thymidine was performed as previously described (Ansar Ahmed et al., 1994). Briefly, 1 μCi of [^{3}H) thymidine was added to each well 48 hours after culture; cells were harvested 24 hours after being pulsed using a PHD harvester (Cambridge Technologies Inc. MA). Radioactive uptake by the filters was determined using a Beckman 8100 LS beta-counter.

Assays to determine apoptosis: Several techniques are available to detect cells undergoing apoptosis, however, all of the assays available have shortcomings (Schmid et al ; McGahon et al., 1995). Therefore, in our study we have utilized several different methods: (i) Qualitative assessment of cell size and granularity by forward scatter and side scatter analysis on the flow cytometer; (ii) Flow cytometric quantitation of the DNA cell content (hypodiploid) by propidium iodide. (iii) Flow cytometric analysis of membrane integrity by uptake of 7AAD, a vital dye; and (iv), microscopic examination of cells for membrane integrity and DNA condensation by staining with acridine orange

and ethidium bromide.

Propidium iodide (PI): Cell cycle analysis of spleen lymphocytes was performed utilizing the propidium iodide method as described (Robinson, 1993). Briefly, 1 x 10^6 fresh or cultured lymphocytes were spun, decanted and then fixed and permeabilized in azide buffer (10 g of SA-bactobuffer, 10 μl of 10% sodium azide and 10% heat inactivated FBS) and stained with a Vindelov's solution (1.21 gm Tris base, 584 gm NaCl, 10 mg RNAse, 50.1 mg propidium iodide, 1 ml Nonidet P-40, pH 8.0) overnight. Propidium iodide stains the DNA in the live cell. Apoptotic cells have lowered staining with PI (sub-G0/G1 peak or Ao cells). Reductions in the DNA content are due to DNA fragmentation and subsequent diffusion of low molecular weight DNA products from the cell, and/or marked condensation of the DNA which makes DNA inaccessible for the dye (McGahon et al., 1995). Staining with PI has the advantage that cell cycle stages can be identified. For example, cells that are synthesizing new DNA (S) or have doubled their DNA content prior to undergoing mitosis (G2/M) can be identified. Disadvantages of this method include: (i) PI has been reported to be unable to identify apoptotic cells in fresh thymocytes (Schmid et al., 1994); (ii), PI staining does not differentiate between early and late apoptosis; and (iii), cells dying by necrosis can not be separated from cells in resting (G0/G1) stage.

Staining of cells with 7-amino-actinomycin D (7AAD): This method utilizes 7AAD to stain unfixed cells (Schmid et al.,1994). Briefly, cells were prepared as described above, resuspended at 5 x 10^6 cells/ml in PBS and then incubated with 100 μl (5 μg/ml in PBS) of 7-AAD for 15 to 30 minutes at 4°C protected from light. Then cells were then analyzed by flow cytometry (Coulter EPICS XL). This method allows for the

differentiation of live cells ($7AAD^{dull}$) from those in early ($7AAD^{intermediate}$) and late apoptosis/necrosis ($7\text{-}AAd^{hi}$).

Acridine orange and ethidium bromide (AO/EB): As described previously (McGahon et al., 1995). Briefly, cells, in 25 μl of RPMI media, were mixed with 1 μl of the dye mixture (100 μg/ml acridine orange + 100 μg/ml ethidium bromide), mixed gently and examined under UV light. At least 200 cells were counted per sample and quantified as described (McGahon et al., 1995). This method employs the differential uptake of fluorescent DNA binding dyes acridine orange and ethidium bromide to determine viability of cells. Ethidium bromide is taken by non-viable cells and intercalates into the DNA making the nucleus appear orange with a dark red cytoplasm. Acridine orange stains viable cells, intercalates into the DNA giving it green fluorescence and stains RNA red. Thus, live cells with intact membranes will have a uniform green color with a thin orange cytoplasm surrounding it. Early apoptotic cells with intact membranes and fragmented DNA have green nuclei with bright green patches in the nuclei reflecting chromatin condensation and an increase in the cytoplasm / nucleus volume ratio. As the cell death progresses and membrane blebbing starts, ethidium bromide enters the cell which now appears orange. Late apoptotic cells have bright orange chromatin condensation areas which distinguishes them from necrotic cells which have uniform orange color (**figure 7.1**).

Statistical Analysis: Non-parametric ANOVA or Mann-Whitney tests were employed were employed to assess differences among treatment groups given the lack of normality of the distribution of the data.

RESULTS:

Splenic lymphocytes from mice treated with estrogen are heterogenous populations: Segments of freshly collected spleens (9 from estrogen-treated mice and 9 from orchiectomized controls) were imprinted on a glass microscope slide, air dried, stained with Wright's stain, and observed under 40X magnification. A differential count of 1000 cells per sample was performed. As shown in **Figure 7.2**, the spleen cells from estrogen-treated mice showed a high degree of heterogeneity. Spleens from mice treated with estrogen had a relative decrease in the number of small resting lymphocytes compared to controls (89.5% and 95.9%, respectively; $p<0.01$). Flow cytometric analysis of freshly isolated splenic lymphocytes indicated that cells from estrogen-treated mice had increased variability in size and density as determined by forward and side scatter analysis **(figure 7.3)** that was not present in spleens from mice treated with placebos.

Spleen lymphocytes from mice treated with estrogen have higher proportions of cells actively dividing and undergoing apoptosis: Freshly isolated lymphocytes from the spleen of mice treated with estrogen had a slightly higher proportion of cells undergoing apoptosis as assessed by acridine orange and ethidium bromide **(figure 7.4a)** or by staining with 7AAD **(figure 7.4b).** Accordingly, the percent of live cells was reduced *ex vivo* in mice that had been treated with estrogen. In agreement with reports in the literature (Schmid et al., 1994), no increase in the hypodiploic fraction of freshly isolated spleen cells was observed utilizing PI stain **(figure 7.4c)**. Interestingly, staining with PI did show a ten fold increase in the proportion of cells in the S and M/G2 phases of the cell cycle. This, together with a slight increase in the number of lymphoblasts (1.16%

and 0.7% in estrogen-treated and placebo-treated mice, respectively; data not shown) suggested that lymphocytes in the spleen were actively proliferating in the absence of an evident stimulus.

Splenic lymphocytes from estrogen-treated mice proliferate in culture in the absence of stimulation: To assess whether estrogen-treatment induced lymphocyte proliferation, lymphocytes from spleen of estrogen-treated and placebo-treated orchiectomized mice were cultured for 72 hours in complete media. Proliferation was determined utilizing the nonradioactive Alamar Blue method. Spleen lymphocytes from estrogen treated mice proliferated significantly more than placebo-treated mice in the absence of exogenous stimulus ($p < 0.05$) **(figure 7.5)**. These experiment was repeated 5 times using 1 - 3 mice per group.

Fresh B cells from estrogen-treated mice have increased proportions of cells dying by apoptosis: The effects of estrogen *in vivo* with regards to cell size and granularity, autoproliferative character, and increased proportion of cells dying by apoptosis were examined in the splenic B cell population. As shown in **Figure 7.6**, as assessed by forward and side scatter flow cytometric analysis purified B cells from estrogen treated mice constituted a heterogenous population in terms of size and granularity.

The presence of cells undergoing apoptosis *in vivo* was assessed by fluorescent microscopy by staining the cells with acridine orange and ethidium bromide, as well as by flow cytometry utilizing 7-AAD and PI. As shown in **Table 7.1**, as assessed by acridine orange and ethidium bromide, 21% percent of the B cells isolated from the spleens of estrogen-treated mice were undergoing apoptosis (15.4% in early apoptosis

and 6.1% in late apoptosis) compared to only 8.5% of the placebo-treated controls (4.8% in early apoptosis and 3.7% in late apoptosis). Apoptosis assessed by staining with 7AAD (**Table 7.1**), revealed more modest differences. Apoptotic cells in spleens from mice treated with estrogen accounted for 16.2% of the cells (4.6% and 11.6% in early and late apoptosis, respectively), compared to 13.5% (2.8% and 10.7% in early and late apoptosis, respectively) in placebo controls. As observed for total spleen lymphocytes, PI analysis of fresh B cells showed a distinctly lower number of hypodiploid cells undergoing apoptosis (**Table 7.1**), (less than 1 % for both groups (0.125 % and 0.275% in mice treated with placebo and estrogen, respectively).

Purified splenic B cells of estrogen-treated mice proliferate spontaneously in culture: To determine whether the autoproliferative abilities of estrogen-treated derived lymphocytes corresponded to the B cell population, we cultured purified B cells in complete media for 72 hours and determined proliferation by the Alamar Blue method. As shown in **figure 7.7,** B cells from mice treated with estrogen proliferated significantly more in culture than those of controls ($p < 0.05$).

Cell cycle analysis confirmed the autoproliferative character of purified B cells from spleen of mice treated with estrogen **(Table 7.1)**. Splenic B cells from mice treated with estrogen had a consistent increase (approximately 9 fold) in the proportion of cells in the S and M/G2 stages of cell cycle.

B cells from estrogen-treated mice resist apoptosis in culture: Splenic B lymphocytes from normal mice have been shown to die by apoptosis *in vitro* unless rescued by stimulation (Illera et al., 1993). Since we had observed an increase in the proportion of

B cells undergoing apoptosis *ex vivo* in mice treated with estrogen, we next determined whether estrogen treatment modified the susceptibility of unstimulated splenic B cell to undergo apoptosis *in vitro*. Unstimulated splenic B cells were cultured in complete media and apoptosis was assessed after 24, 48 and 72 hours. As assessed by PI, increased percentages of B cells were hypodiploic (apoptotic) as early as 24 hours, and differences were dramatic at 48 hours of culture **(Figure 7.8a).** Similarly, decreased numbers of unstimulated B cells at 48 and 72 hours of culture were apoptotic in estrogen-treated mice (73%) compared placebo-controls (83%) as determined by acridine orange and ethidium bromide (**figure 7.9a**). Comparable reduction in the susceptibility to apoptosis was observed in B cells stained with 7-AAD (**Figure 7.10.a**)

Stimulation with LPS and anti-CD40 antibodies elicits a normal proliferative response in mice treated with estrogen: Since spleen lymphocytes from estrogen treated mice had increased proliferation in the absence of stimuli, we next investigated the proliferative response to specific B cell stimulants. Spleen cells were cultured in the presence of a T-cell independent antigen, LPS (10 and 25 μg/ml) and in the presence of anti-CD40 (5 μg/ml) alone or together with IL-4 (10 and 100 U/ml) for 72 hours and proliferation was assessed by the Alamar Blue assay. As shown in **figure 7.7,** the proliferative response of B cells to stimulants was increased compared to controls. However, when the background (spontaneous proliferation in complete media) was subtracted, no differences were observed between mice treated with estrogen and controls. This indicated that despite the autoproliferative character induced by estrogen, B cells were able to respond adequately to stimulation. Stimulation of B cells with IL-4 alone (10 to 100 U/ml) had no significant proliferative effect (data not shown).

Splenic B cells from estrogen-treated mice show increased viability upon stimulation: Splenic B cells were stimulated *in vitro* with LPS (10 μg/ml and 25 μg/ml), and with anti-CD40 alone or in combination with IL-4 (10 to 100 U/ml). The presence of apoptotic cells was assessed after 24, 48 , and 72 hours by staining with PI, acridine orange and ethidium bromide, and 7-AAD. Experiments were repeated 4 times with consistent results. A representative example is illustrated in **figures 7.8, 7.9 and 7.10.** Purified B cells from mice treated with estrogen, stimulated with LPS (10 μg/ml), had reduced numbers of cells undergoing apoptosis after 48 and 72 hours inn culture. Similar results were obtained by acridine orange and ethidium bromide **(figure 7.9b)**, and 7-AAD **(figure 7.10b).** Stimulation of B cells with anti-CD40 antibodies, has been shown to rescue cells from apoptosis. Lymphocytes from estrogen-treated mice stimulated with antibodies to CD40 had reduced the percentage of cells undergoing apoptosis from 26% (in media) to 4 % (stimulated with anti-CD40 antibodies) at 48 hours in culture as assessed by PI. In contrast, similar cultures from placebo-treated mice reduced apoptosis from 86% in media, to 54% in anti-CD40 stimulated cultures (**figure 7.8c**). Similar results were obtained by PI and 7AAD (**figures 7.9 c and 7.10 c**). Stimulation of lymphocytes with antibodies to CD40 combined with IL-4, have been shown to rescue cells from apoptosis and has been used to create long term cell cultures with human lymphocytes. After 72 hours, B cells from estrogen-treated mice that were stimulated in this way, had markedly reduced the number of cells dying by apoptosis to 8.2 % as assessed by PI, 14% as assessed by acridine orange and ethidium bromide, and 19% by 7AAD compared to 51%, 59%, and 30% by the three methods respectively, in placebo controls **(figures 7.8d , 7.9d and 7.10d)**.

DISCUSSION:

Our recent experiments had shown that administration of estrogen to nonautoimmune C57BL/6 and BALB/c mice induces the expression of hetero- and autoantibodies that have been associated with autoimmune and infectious diseases (Ansar Ahmed and Verthelyi, 1993; Ansar Ahmed et al., 1993; Verthelyi and Ansar Ahmed, 1994; Verthelyi, Schurig and Ansar Ahmed, unpublished observations). Further, at the cellular level, we had shown that estrogen induced B cell hyperactivity and an increase in the number and activity of plasma cells in the spleen, including those that secrete autoantibodies (Verthelyi and Ansar Ahmed, 1996).

In these studies we have further investigated the effects of estrogen-treatment on the immune function of normal mice by assessing the cell survival and proliferation of splenic lymphocytes *in vivo* and *in vitro* in the presence and absence of stimulation. We have shown that *in vivo* administration of estrogen induces significant changes in the splenic lymphocyte population, including a reduction in the proportion of mature resting B cells, and increased variability of cell size and granularity as assessed by flow cytometry. Reduced size and granularity have been associated with apoptosis (McGahon et al, 1995). Accordingly, spleen cells freshly isolated from mice treated with estrogen had a higher proportion of cells dying by apoptosis. Further, cell cycle analysis indicated that spleen lymphocytes from mice treated with estrogen also appeared to have increased numbers of cells that are actively dividing. Similar results were obtained in purified B cells. It is important to point out that the total number of lymphocytes in the spleen as well as the proportion of B cells, in estrogen-treated mice is not increased (but rather decreased), Therefore the increase in proliferation and apoptosis observed *in vivo,* in

mice treated with estrogen, appears to be due to an estrogen-induced increase in lymphocyte turnover. In other words, it is possible that estrogen could be activating a group of B cells to enter cell cycle, and then the activated lymphocytes would die in the absence of further stimulation.

To further explore the estrogen-induced changes on the B cells of mice treated with estrogen, we assessed the response of B cells in culture in the absence of obvious stimulants. We determined that B lymphocytes from mice treated with estrogen were able to avoid death and proliferate in culture in the absence of stimulation. Interestingly, this is unlike lymphocytes from MRL/lpr mice, which were also shown to have increased numbers of cells undergoing apoptosis *in vivo,* but have accelerated apoptosis *in vitro* in the absence of stimulation (Emlen et al., 1994).

We next investigated the response of B cells to stimulation in culture, and found that despite the autoproliferative character of B cells from mice treated with estrogen, upon stimulation, B cells were able to mount a proliferative response comparable to that of placebo controls. Stimulating B cells with LPS and antibodies to CD40 also reduced the proportion of B cells that underwent apoptosis in culture. Our results suggest that the estrogen acted synergistically with LPS and/or anti-CD40 antibodies to block B cells from undergoing apoptosis. Together these results demonstrate that exposure to estrogen *in vivo* alters B cells function to allow for spontaneous proliferation, and reduced susceptibility to death by apoptosis.

Under normal circumstances the expression of autoantibodies is tightly regulated by several methods including clonal deletion. In autoimmune diseases the expression of

autoantibodies has been linked to defects in clonal deletion mediated by apoptosis. Estrogen may act on the B cells of nonautoimmune mice to skew the expression of genes that regulate apoptosis. For example, estrogen could induce a higher baseline level of proteins that block apoptosis such as Bcl/2 or Bcl/x, or reduce the surface expression of Fas. Intrinsic higher baseline levels of Bcl/2 could block the deletion of unstimulated B cells at the germinal center allowing self-reactive B cells to survive and undergo isotype switch and eventually become memory B cells. These aspect are currently under investigation in our laboratory. Alternatively estrogen could also induce increased levels of Bcl/2 or reduced expression of Fas antigen on activated B cells. This would obstruct the prompt elimination of activated B cells after antigen-driven activation, and could potentially lead to the expression of autoreactive B cells. Another marker that has been associated with apoptosis in B cells is leukosialin or CD43. Surface expression of CD43 has also been shown to reduce B cell susceptibility to undergo apoptosis (Dragone et al., 1995). These aspects need to be further investigated.

We have shown that exposure to normal mice to moderate levels of exogenous estrogen modifies B cells function *in vivo* to alter the B cell repertoire, increase B cell activity, including induction of B cell proliferation (both *in vivo* and *in vitro)*, and reduction of their susceptibility to die by apoptosis *in vitro*. It is unknown at this time whether the autoproliferative character of the lymphocytes or their resistance to undergo apoptosis are associated with B cell hyperactivity or the production of autoantibodies. Further experiments to characterize this population are warranted.

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from the Lupus Foundation of America. We thank Dr. Maureen Howard from DNAX Research Institute for generously providing the anti-CD40 antibody. We thank Dr. Chickering for his expert performance of the differential counts of splenic leukocytes; Mrs Jane Walsh for her invaluable help with the isolation and purification of lymphocytes.

666Table 1: Effects of exposure to estrogen on splenic B cells *ex vivo*.

Acridine orange and ethidium bromide:

MICE	LIVE (mean ± SEM)	EARLY APOPT. (mean ± SEM)	APOPTOTIC (mean ± SEM)	NECROTIC (mean ± SEM)
P-O	84 ± 2	4.8 ± 1	3.7 ± 2.9	9.7 ± 1.6
E2-O	67.7 ± 7	15.4 ± 3	6.1 ± 1.7	11 ± 2.3

7-amino-actinomycin D

MICE	LIVE (mean ± SEM)	EARLY APOPT. (mean ± SEM)	LATE APOPT. (mean ± SEM)
P-O	85 ± 3.5	2.8 ± 0.5	10.7 ± 2.8
E2-O	83.8 ± 2.5	4.6 ± 0.7	11.6 ± 2.3

Pripidium iodide:

	HYPODIPLOID	G0/G1	S	M/G2
P-O	0.125 ± 0.01	95.9	0.65	0.49
E2-O	0.275 ± 0.02	86.95	5.45	4.23

Apoptosis of freshly isolated splenic B cells was assessed by three independent methods. Results represent mean and SEM of at least 4 independent experiments.

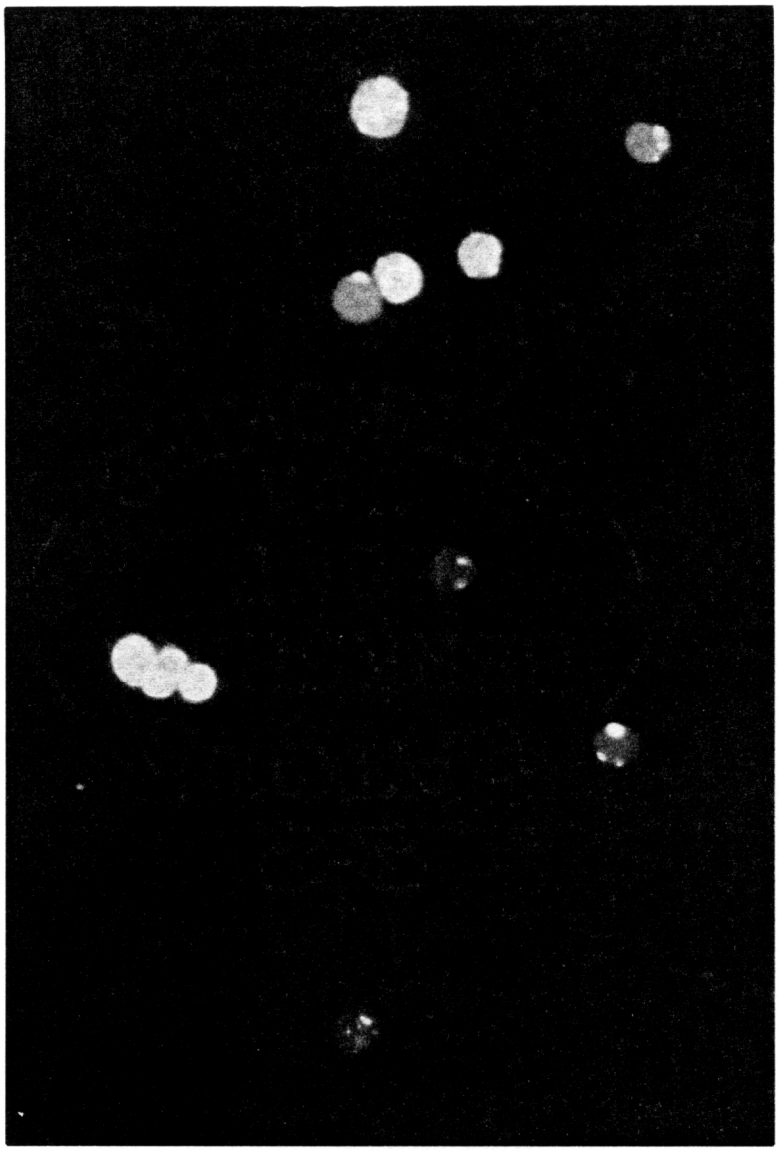

Figure 7.1: Freshly collected spleen cells from mice treated with estrogen and controls stained with acridine orange and ethidium bromide. 40X magnification.

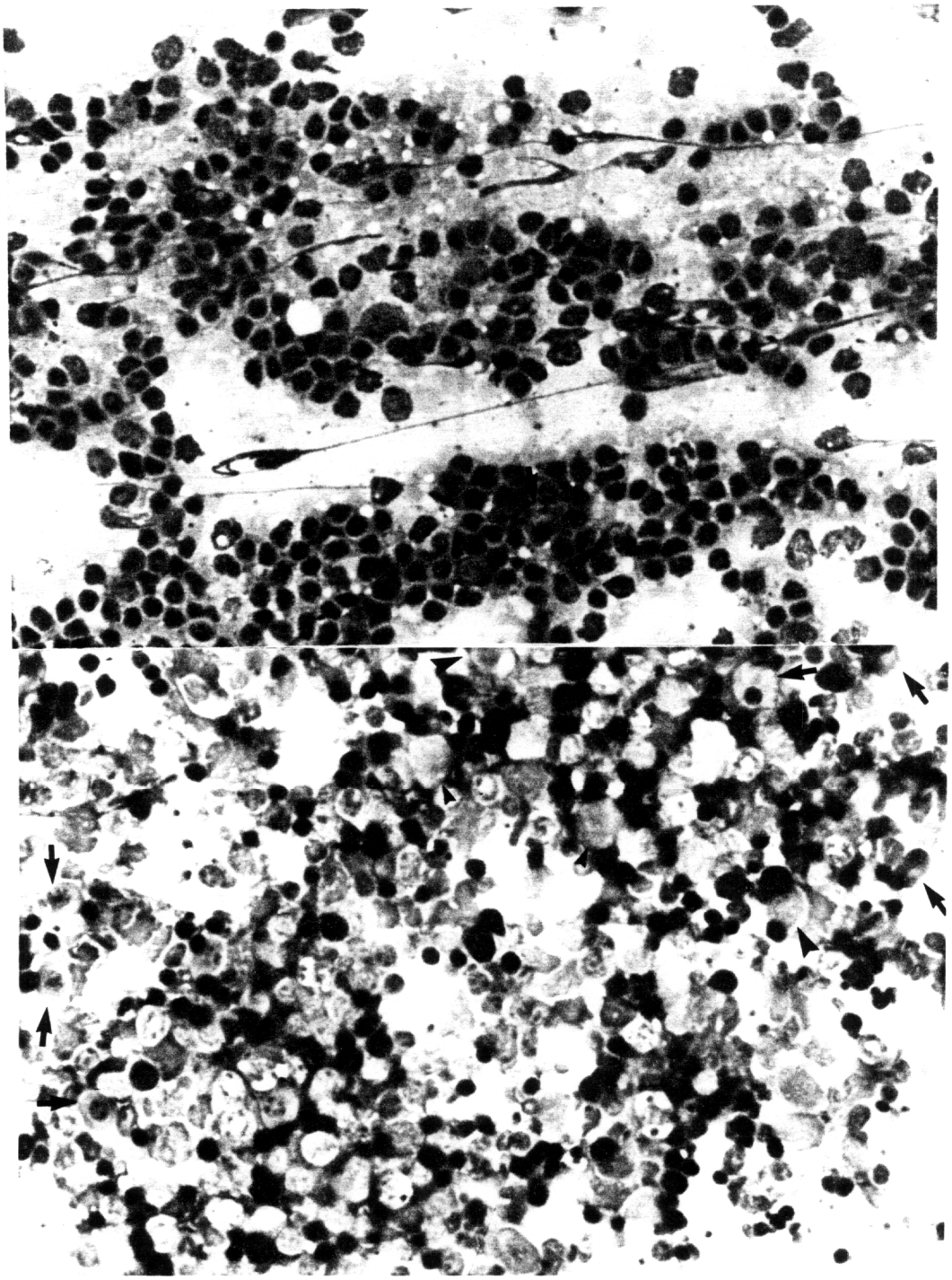

Figure 7.2: Spleen cells from mice trated with estrogen and controls, stained with Wright's stain. 200X magnification. Note the morphological heterogeneity of cell in the spleen from mice treated with estrogen.

P-O

E2-O

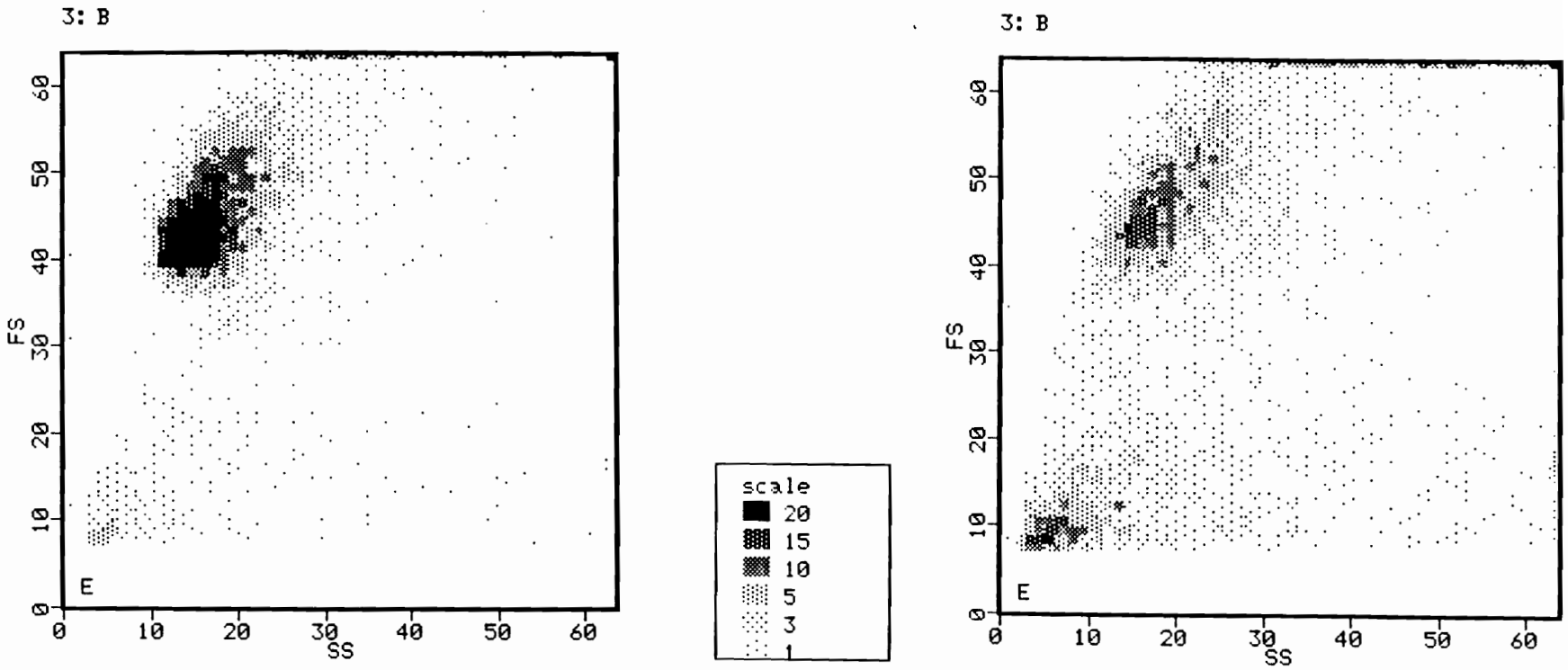

Figure 7.3: Size and granularity of freshly collected spleen cells from placebo and estrogen treated mice as assessed by acridine flow cytometry

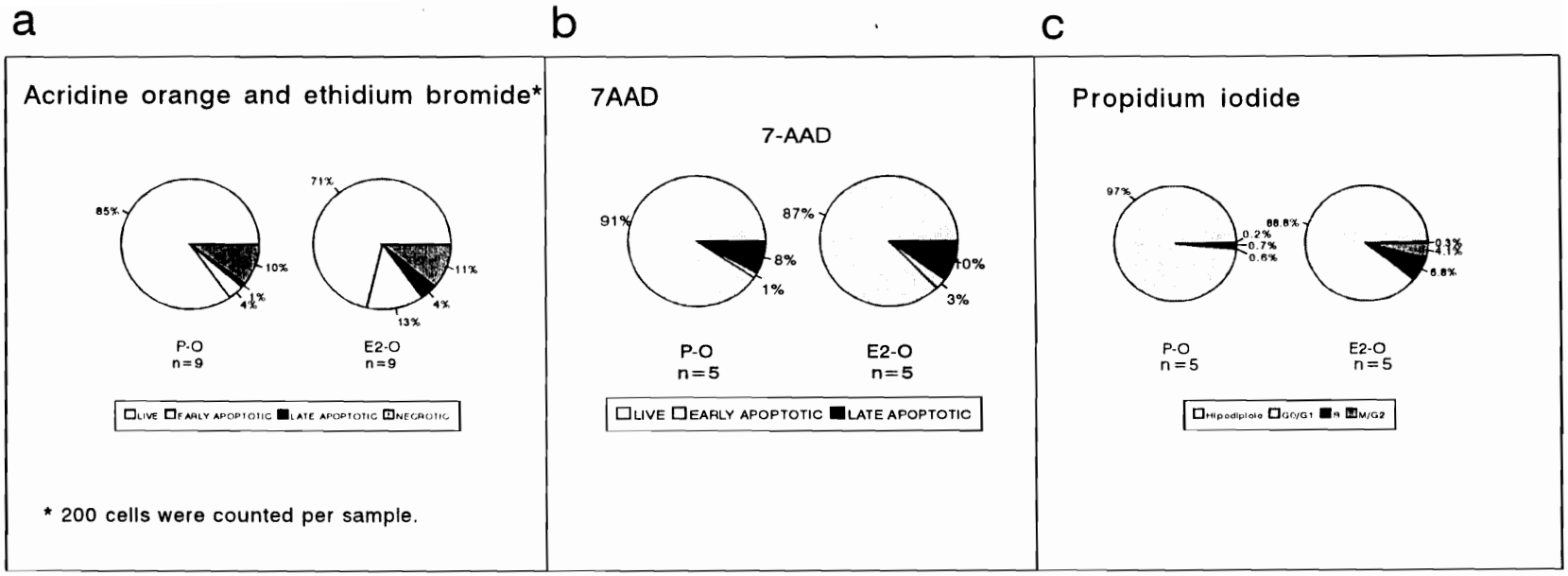

Figure 7.4. Freshly isolated spleen lumphocytes from placebo or estrogen-treated C57BL/6 mice were assessed for viability and cellular death by apoptosis. The staining and analysis procedures as described in materials and methods section.

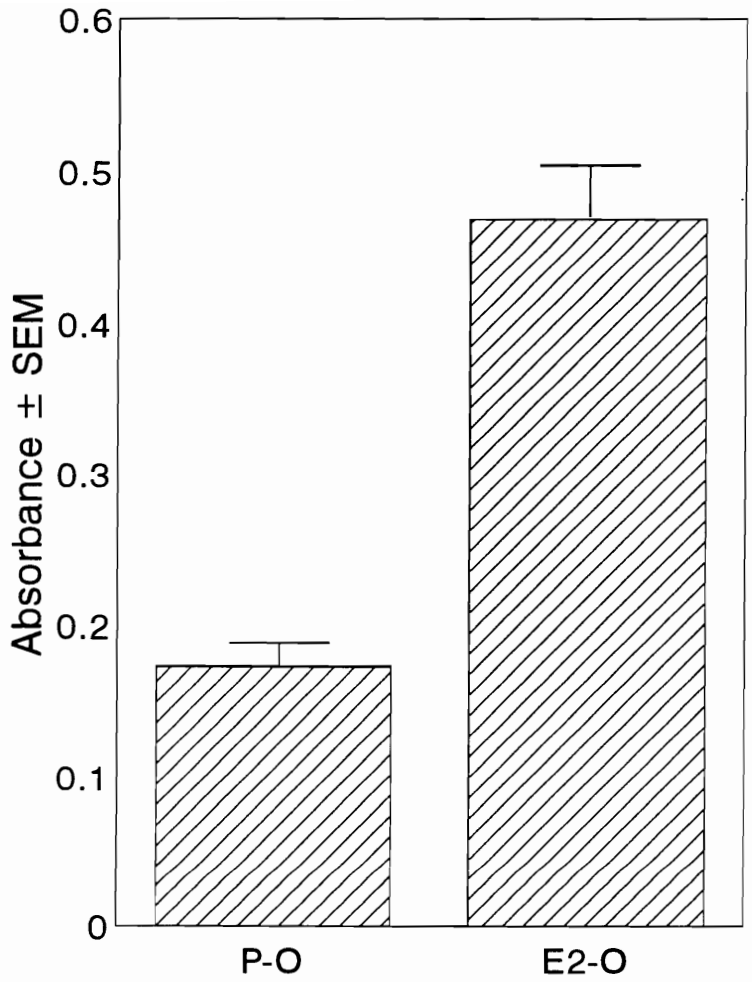

Figure 5: Proliferation of spleen lymphocytes in the absence of stimulation was assessed by Alamar Blue as described in materials and methods. Values represent the mean and SEM of 5 experiments. Lymphocytes from mice treated with estrogen proliferate significantly more than thos from control mice ($p<0.05$) as tested by Mann Whitney non parametric test.

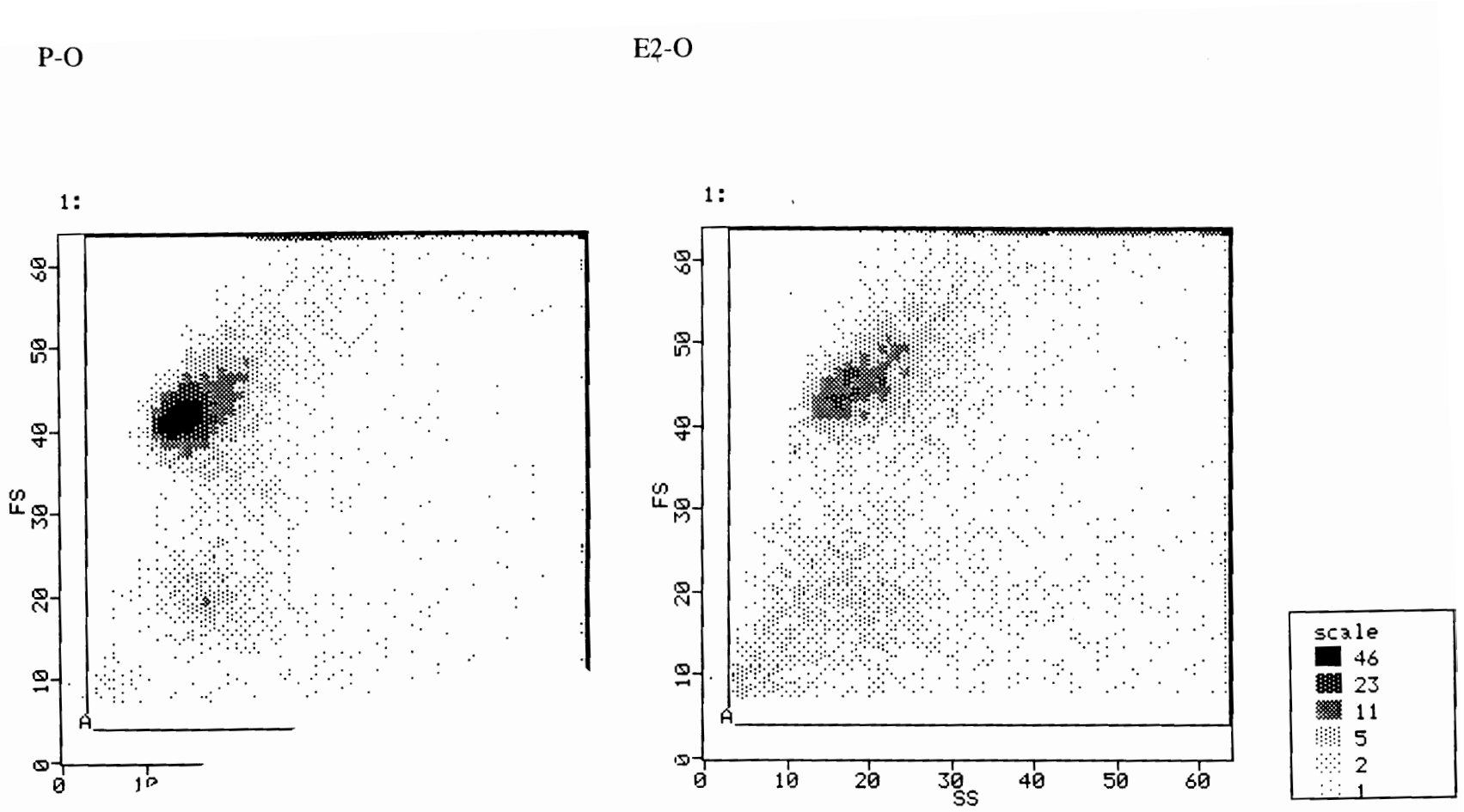

Figure 7.6: Size and granularity of freshly collected spleen B cells from placebo and estrogen treated mice as assessed by flowcytometry.

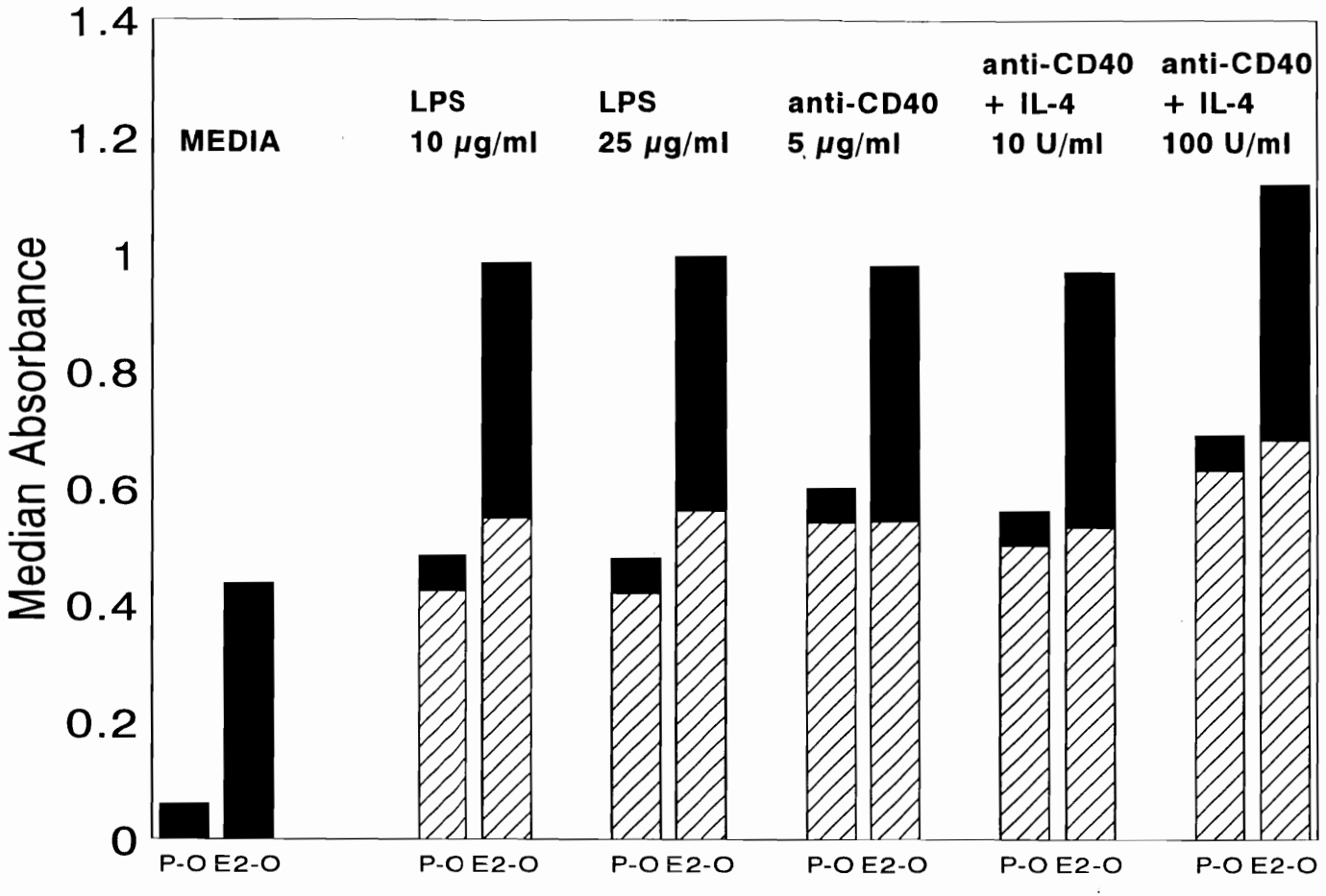

Figure 7.7: Proliferation of spleen B cells in media response to stimulation was assessed by Alamar Blue as described. Values represent the median of 7 experiments. Lymphocytes from mice treated with estrogen proliferate significantly more than those from control mice ($p<0.05$) s tested by Mann Whitney non parametric test. Note, however, that the difference in proliferation in response t stimulation is minimal(hatched bars) (p=ns) if the absobance corresponding to spontaneous proliferation is subtracted (full bars).

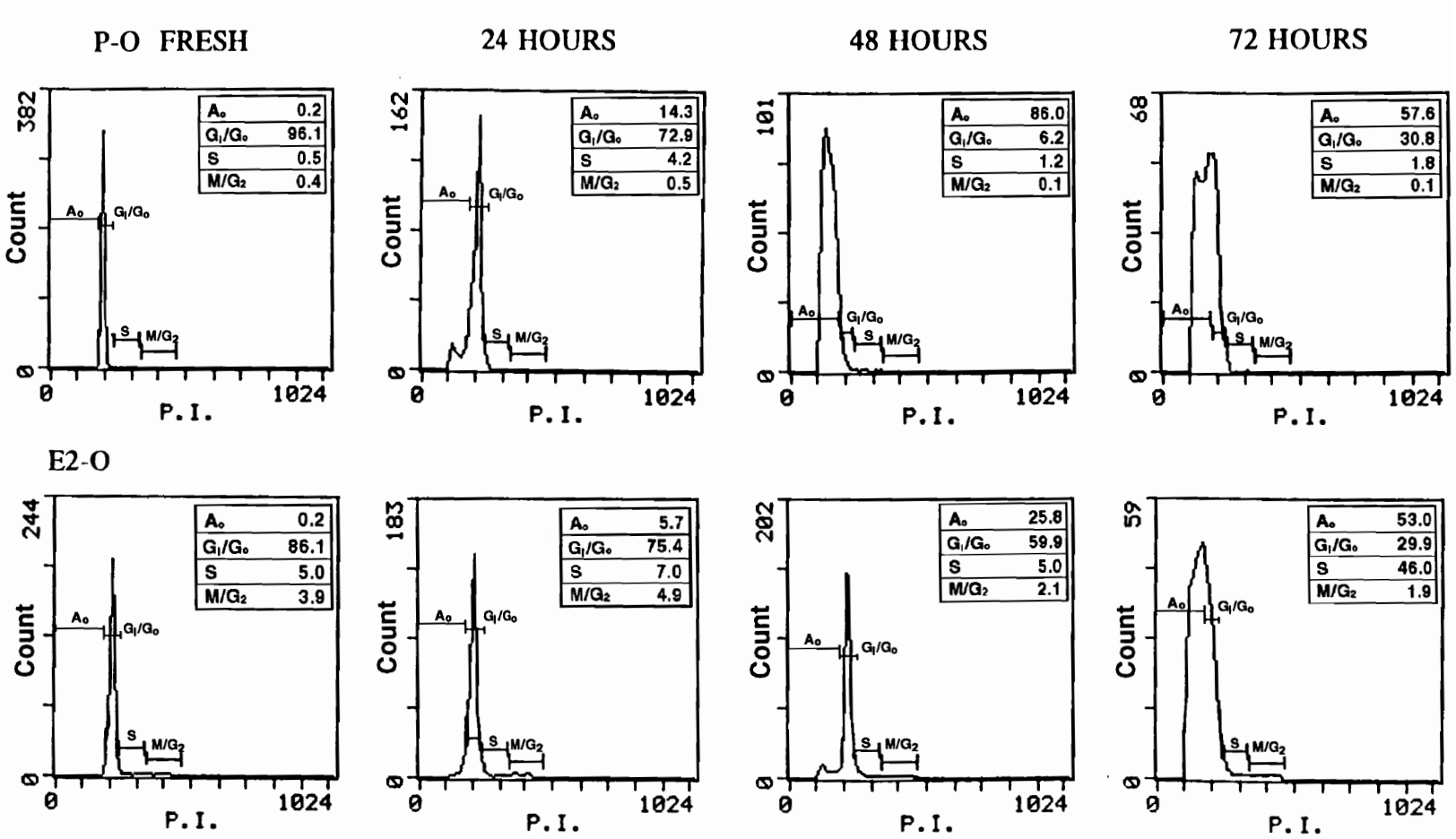

Figure 8a: Apoptosis of unstimulated B cells in complete media as assessed by PI.

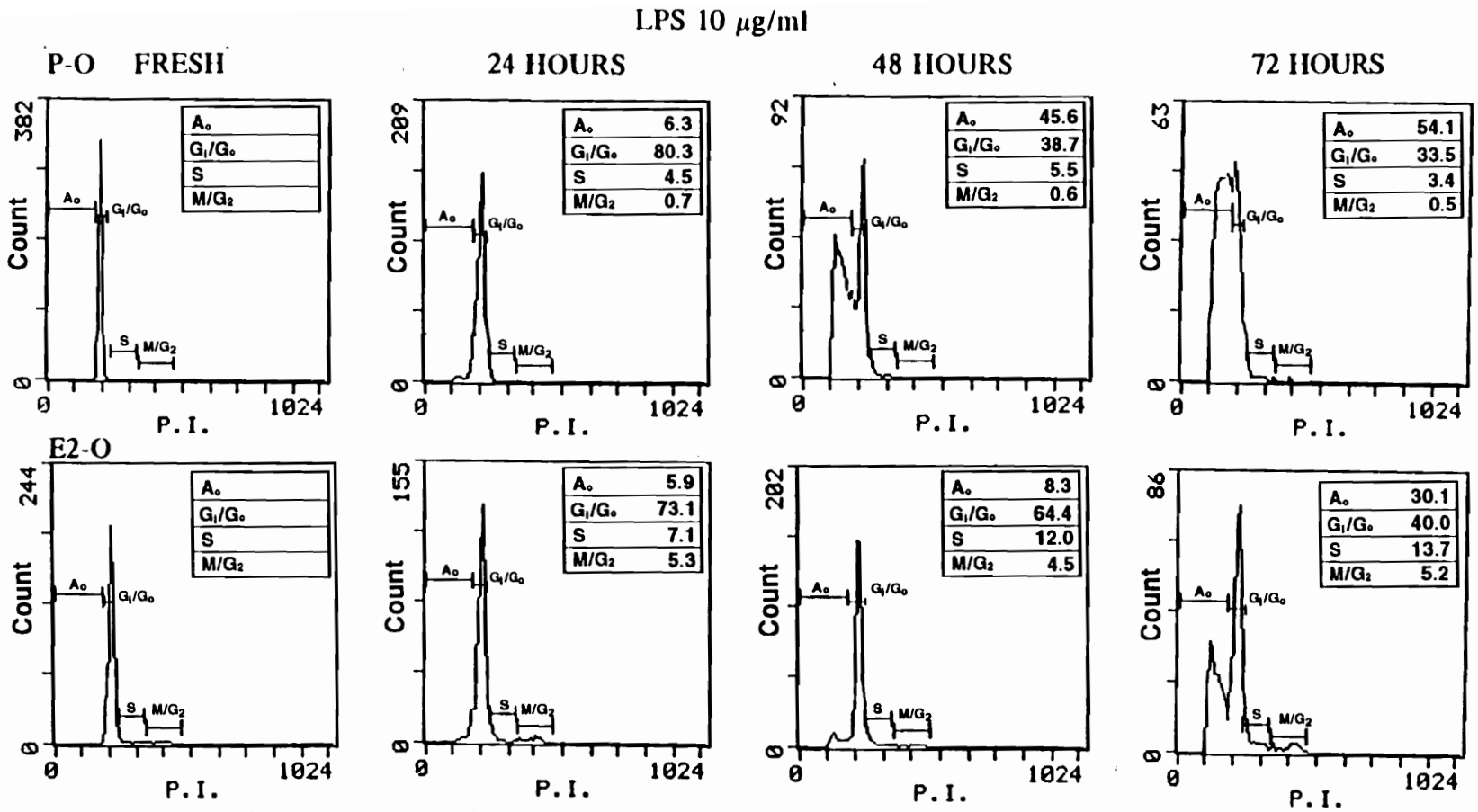

Figure 8b: Apoptosis of B cells stimulated with LPS (10 ug/ml) as assessed by PI.

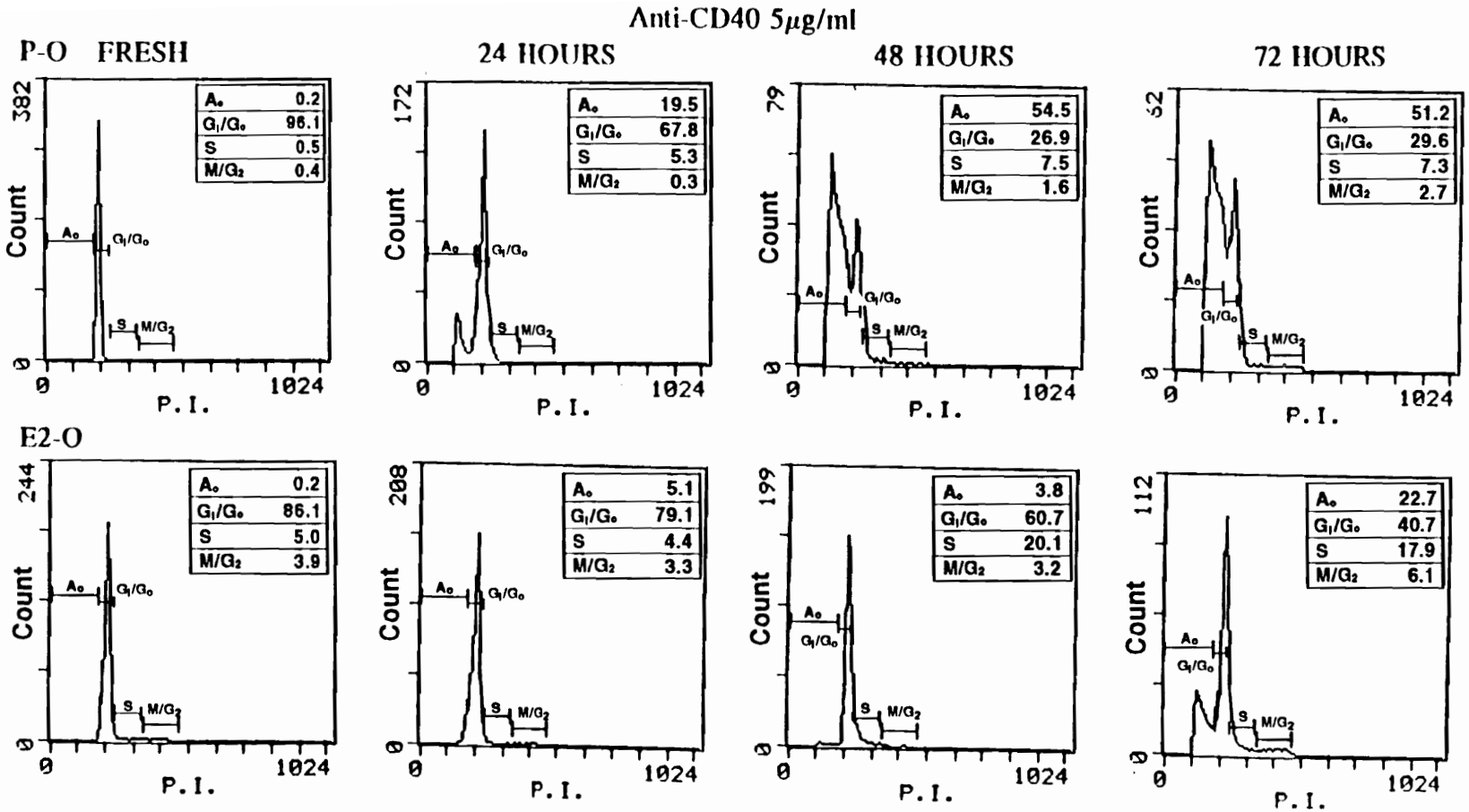

Figure 8c: Apoptosis of B cells stimulated with anti-CD40 antibodies (5 ug/ml) as assessed by PI.

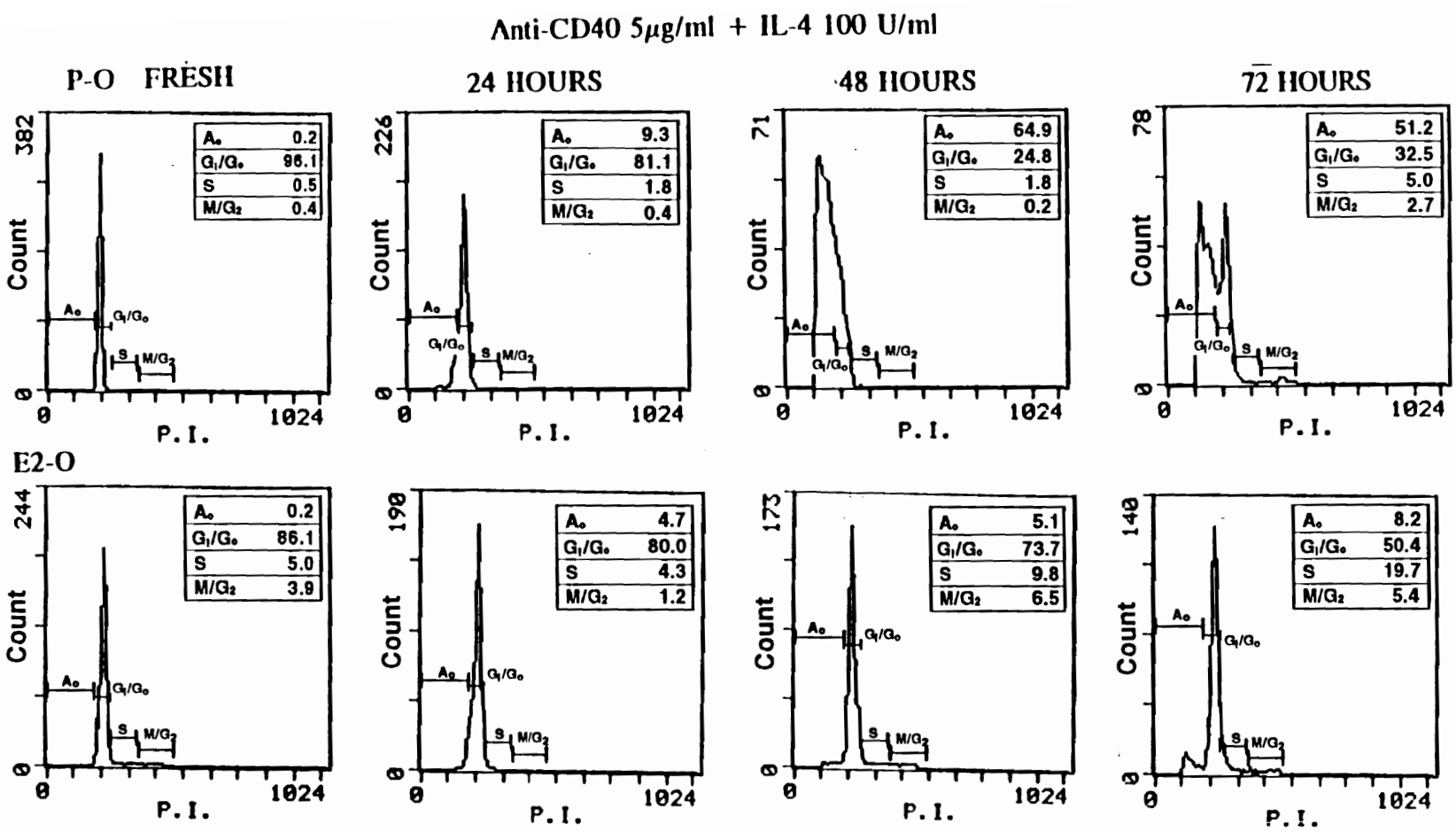

Figure 8d: Apoptosis of B cells stimulated with anti-CD40 antibodies + IL-4 (100U/ml) as assessed by PI.

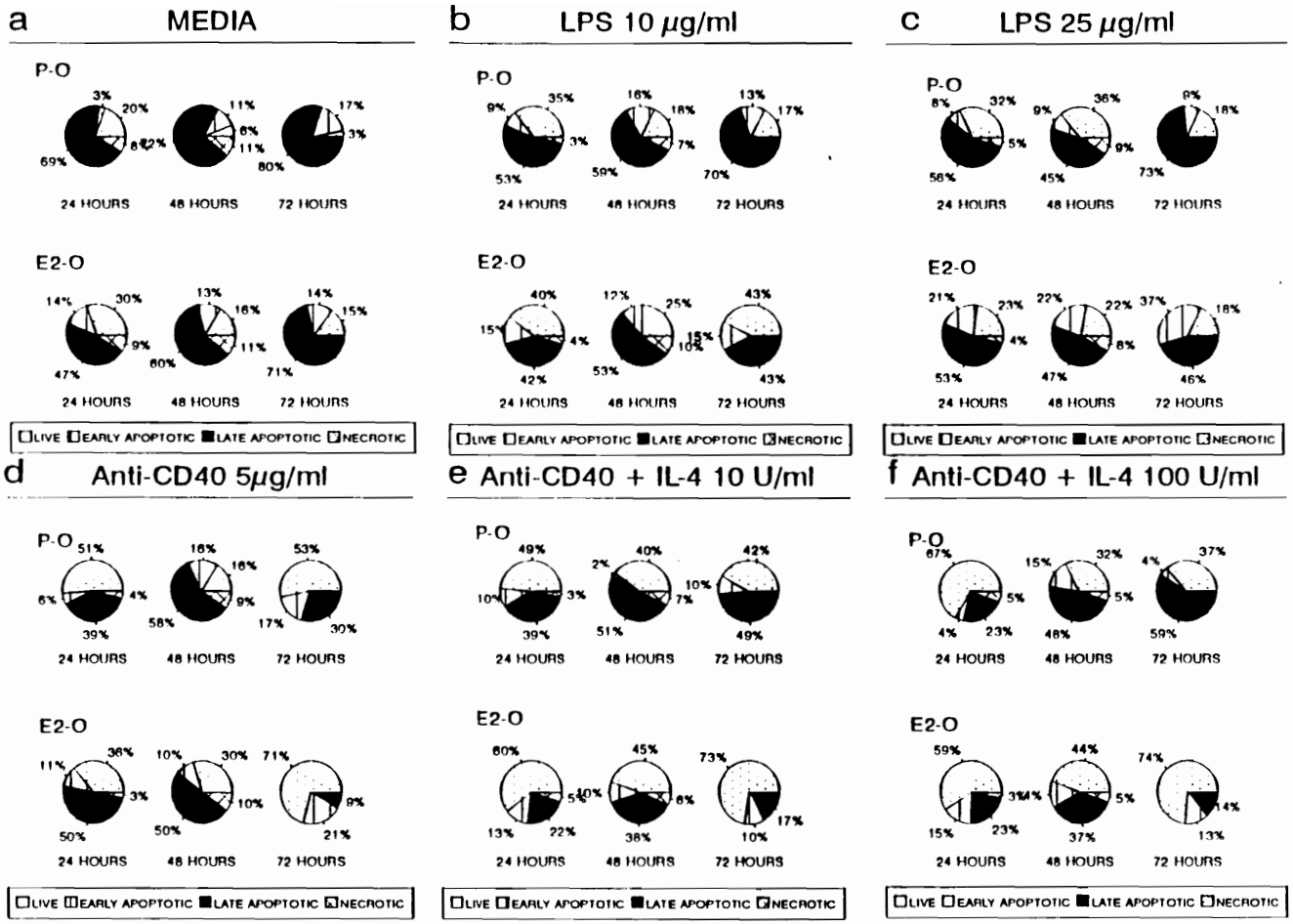

Figure 9:Cell death by apoptosis of splenic B cells was determined by acridine orange and ethidium bromide as described in materials and methods. two hundred event were counted per sample. This is a representative example of 5 independent experiments.

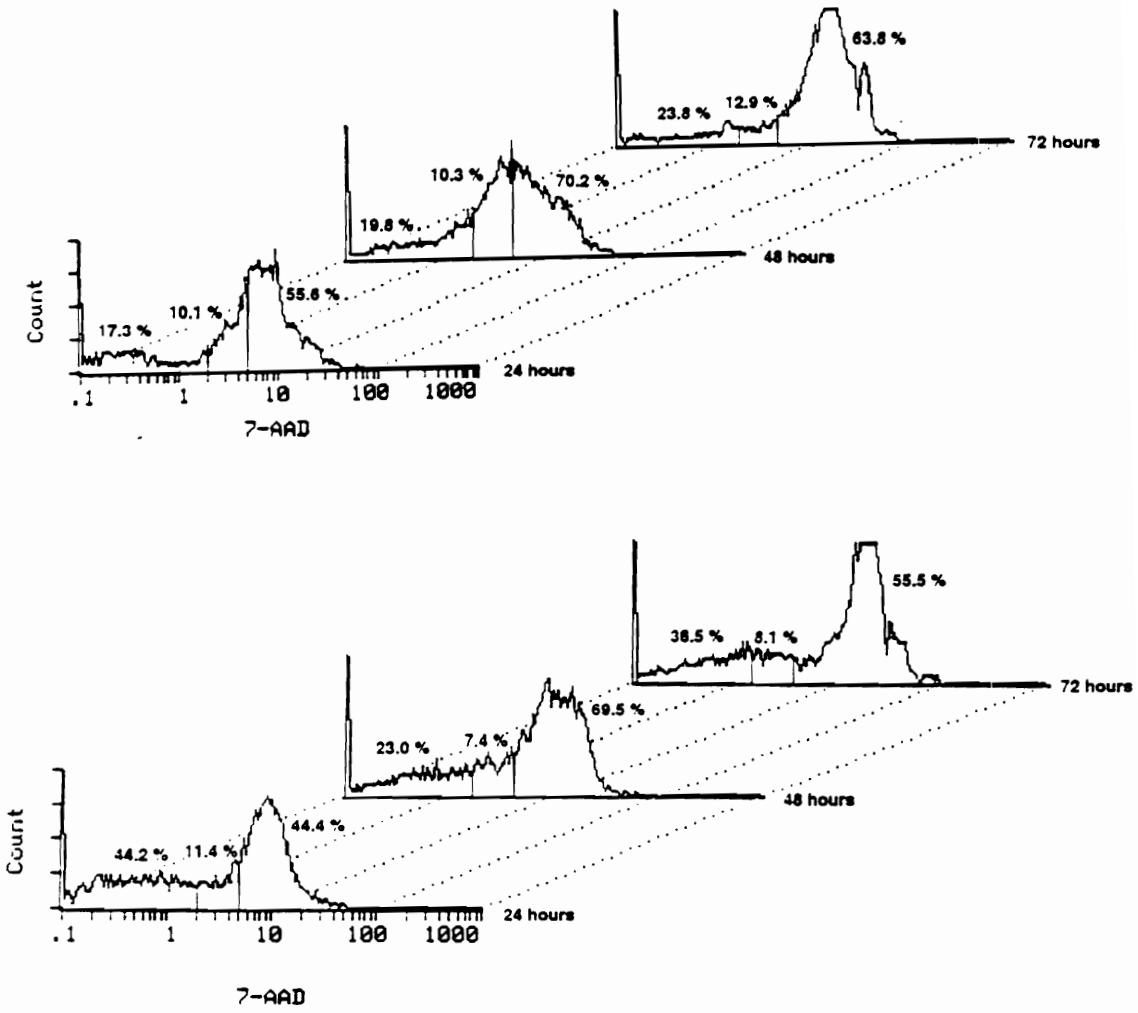

Figure 10a: Apoptosis of unstimulated B cells as assessed by 7-AAD.

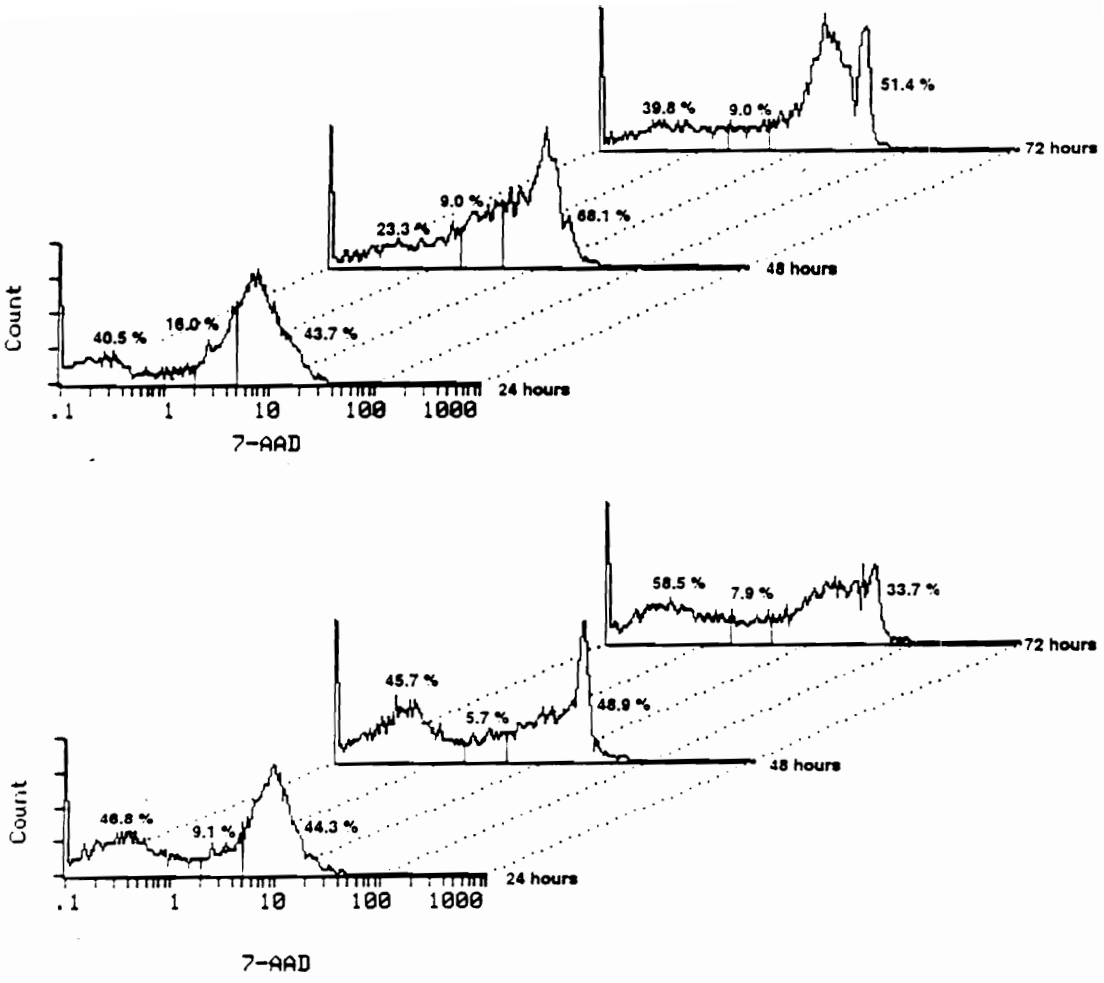

Figure 10b: Apoptosis of B cells stimulated with LPS (10ug/ml) as assessed by 7-AAD.

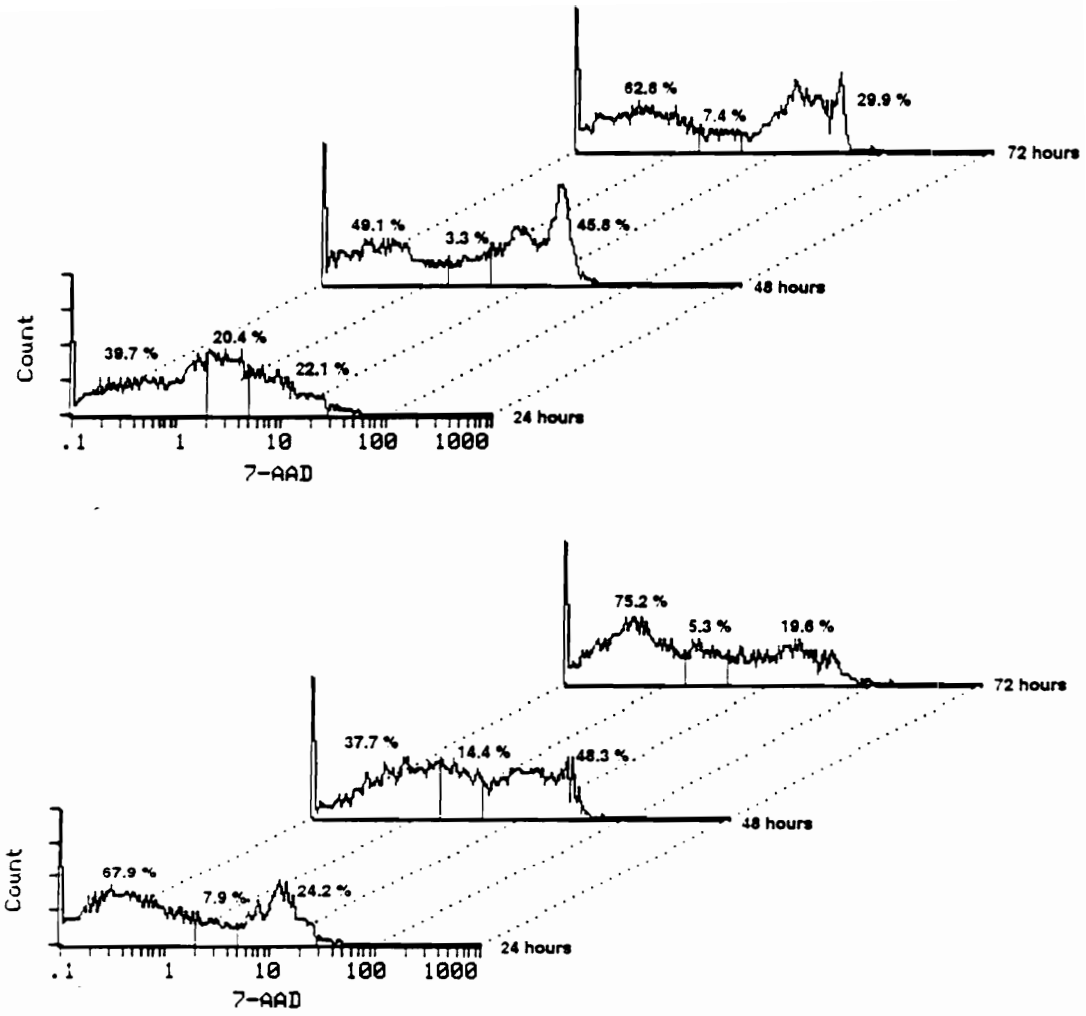

Figure 10d: Apoptosis of B cells stimulated with anti-CD40 antibodies + IL-4 (100U/ml) as assessed by 7-AAD.

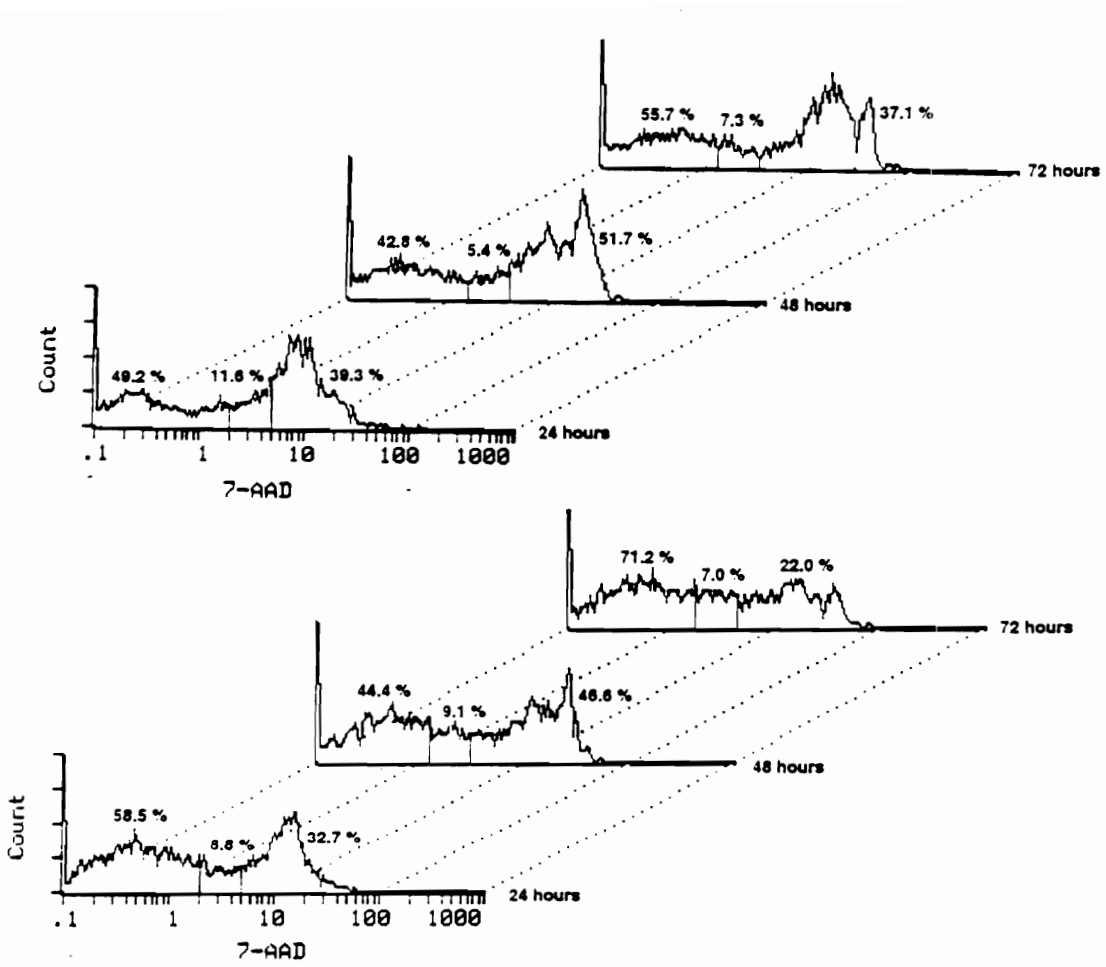

Figure 10c: Apoptosis of B cells stimulated with anti-CD40 antibodies (5ug/ml) as assessed by 7-AAD.

CHAPTER 8:

ESTROGEN INDUCES ANTIBODIES TO *BRUCELLA ABORTUS* STRAIN RB51 IN BALB/c MICE: Characterization of the immune response to *Brucella* in nonautoimmune mice treated with estrogen

INTRODUCTION:

Over the past years, evidence has shown that there are marked gender differences in immune capabilities. Women have been shown to have stronger immune capabilities (particularly antibody-mediated) than their male counterparts (reviewed in Ansar Ahmed et al, 1985a; Schuurs and Verheul, 1990; da Silva, 1995). This allow them to respond better to most microbial (eg. *E.coli*, Brucella, measles, rubella and hepatitis B) and non-microbial antigens than their male counterparts (Ansar Ahmed et al, 1985a; Schuurs and Verheul, 1990; Grossman, 1985; Kenny and Gray, 1971). The greater immune responsiveness in women is also evident in their higher susceptibility to autoimmune diseases, especially during their reproductive years (reviewed in Ansar Ahmed et ál., 1985a; Grossman, 1991). Sex hormones are thought to play an important role the regulation of the immune function.

Gender differences in immune capabilities have also been studied in a number of experimental laboratory animals (reviewed in Ansar Ahmed et al, 1985; Grossman, 1985; Schuurs and Verheul, 1990; Ansar Ahmed and Talal, 1991). In normal mice, analogous to the human situation, females have been reported to have higher total immunoglobulin levels, mount a stronger response to vaccination, and produce more antibodies to a variety of hetero- and self- antigens compared to males (Ansar Ahmed et al.,1989b; Ansar Ahmed and Talal, 1990). Reports on the cell mediated immune capabilities of male and female mice are diverse. Female laboratory animals have been reported to have reduced incidence of tumors and reject allografts more rapidly than males (reviewed in Ansar Ahmed et al.,1985; Ansar Ahmed and Talal, 1991). On the other hand, the immune response to intracellular parasitic infection with

Schistosoma mansoni was stronger in males than in female CBA/J mice (Eloi-Santos et al., 1992). Also, castrated C57BL/6J, C3H, and DBA/1 mice that were treated with estrogen had reduced delayed type hypersensitivity with increased levels of antibodies (Myers and Petersen, 1985; Calsten et al., 1989).

We have studied the effect of sex hormones on the immune system of C57BL/6J mice which are currently considered as a non-autoimmune strain (Ansar Ahmed, 1994). In these mice we have shown that treatment with estrogen, induced the expression of a wide variety of IgG and IgM auto- and hetero-antibodies (antibodies to cardiolipin, dsDNA and acetone-killed *Brucella abortus* strain RB51), which have been associated with autoimmune and infectious diseases (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994; Verthelyi and Ansar Ahmed, 1996). At the cellular level, we have show that estrogen increases the number of splenic antibody producing cells (APC) (Verthelyi and Ansar Ahmed, 1996). Further, we have shown that splenic lymphocytes from estrogen-treated C57BL/6 mice proliferate *in vitro* in the absence of stimuli. Purified spleen B cells, however, were shown to be able to proliferate in response to mitogens to a similar extent as the controls. Purified spleen T cells of the same mice, however, were found to have a diminished response to T cell stimulants (Ansar Ahmed, unpublished observations). Kinetic studies revealed that effects of estrogen on the immune system are sustained for months after the exposure to exogenous estrogen has been terminated.

The effects of estrogen on B cells, in our previous studies, were performed exclusively on nonautoimmune C57BL/6 mice. Therefore the objective of this study was to determine whether estrogen has similar antibody-promoting effects on another

nonautoimmune strain of mice, BALB/c. Since estrogen modulates the immune system we investigated whether estrogen alters the response to infection. To accomplish this we vaccinated mice that had been exposed to estrogen with RB51, a viable rough attenuated strain of *B. abortus* (Schurig et al.,1991).

MATERIALS AND METHODS:

Mice: Three week old female BALB/c mice were purchased from Charles River Breeding Laboratories Inc. (Kensington N.Y.). Mice were fed on a commercial diet, and housed in standard cages (3 to 5 mice/cage) with a 14 light/10 dark hours schedule.

Animal model:

Thirty six prepubertal (5 week-old) female Balb/c mice were randomly assigned to two treatment groups: The first group (E n=18) received subcutaneous estrogen implants made with medical grade silastic tubing, the second group (P n=18) received similar, yet empty (placebo), silastic implants (Dow Corning Co., MI). Estrogen implants (7mm long) were prepared to hold 4-6 mg of 17β-estradiol (E2; Sigma, St. Louis, MO) as described (Ansar Ahmed et al., 1986, Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1994) and have been shown to release hormones slowly for several months (Ansar Ahmed et al., 1989). At the time of removal of the implants (1 month) 50% of the estrogen-treated mice showed signs of estrogen effects (distended bladders and urinary incontinence). Two weeks after terminating the hormonal treatment, the mice were bled, and were vaccinated either with viable

Brucella strain RB51 (1 x 10^8 to 1 x 10^9 colony forming units (c.f.u.) or saline in Freunds adjuvant **(Table 1)** (Schurig et al., 1991). After the clearance of the infection (5 weeks post immunization; Schurig et al., 1991), the mice were terminated. Sera and spleen cells were collected.

Serum collection: Serum from each mouse was collected retroorbitally at the time of vaccination and of termination, and kept frozen at -70°C until tested.

ELISA assays:

Antibody levels to RB51 were assessed by coating 96-well high binding ELISA plates with acetone-killed *Brucella abortus* strain RB51 (2 μg/ml) overnight at 4°C. After washing with PBS, the wells were blocked with PBS containing 2% bovine serum albumin (BSA; 75 μl/well) for 90 minutes, and then washed three times with PBS. The serum samples (1:100 dilution in PBS 1% BSA) were incubated in the wells for 3 hours at room temperature. After washing, 50 μl of alkaline phosphatase conjugated goat anti-mouse heavy-chain specific immunoglobulin were added and the plates were further incubated for 1 hour at 37°C in a humid chamber (enzyme-conjugated antibodies from Caltag, South San Francisco, CA, were used in sequential studies; antibodies from Southern Biotechnologies Assoc. were utilized in all other studies). Plates were washed again and then developed with p-nitrophenylphosphate (Sigma, MO) in diethanolamine buffer (pH 9.8), and read after 60 minutes (405 nm, Molecular Devices, Menlo Park, CA). Negative controls included wells with known negative sera, and wells with no sera ("no serum blanks"). The absorbencies utilizing known negative sera were similar to that of "no serum blanks", therefore,

"no serum blanks" were utilized to control for background. In addition, a known high titered sera from BALB/c mice that had been vaccinated repeatedly with RB51 were included on each plate as a positive control for the presence of antibodies to RB51; and an MRL/lpr sera was used as a positive control for antibodies to cardiolipin and dsDNA. Absorbencies are expressed as specific OD which are obtained by: (mean OD of sample *minus* mean OD of "no serum blanks" on the plate). (Ansar Ahmed et al., 1993; Verthelyi and Ansar Ahmed 1994).

Other antibodies: Antibodies to cardiolipin were assessed by standard ELISA as described previously (Ansar Ahmed and Verthelyi, 1993). Briefly, medium binding 96-well flat bottom plates (Costar, Cambridge, MA) were coated with 30 μl/well (50 μg/ml of cardiolipin in ethanol) and left uncovered overnight in the dark at 4°C. The sera were diluted 1:100 in PBS-1% BSA. to avoid the potential interference of bovine β2-glycoprotein I (which is thought to be part of ABS) in the assay. Antibodies to dsDNA (70 μg/ml in tris EDTA, ph 8.0), actin (1 μg/ml coating buffer), ovalbumin (5 μg/ml in PBS), were coated onto high binding ELISA plates. Plates used to assess antibodies to dsDNA were coated with methylated bovine serum albumin (BSA) for 6 hours at 4°C prior to the addition of the S1 nuclease treated dsDNA. The rest of the assay was performed as described above.

Isolation of lymphocytes: Spleens were collected under sterile conditions, gently minced and passed through a wire mesh to produce a single cell suspension. Cells were then washed twice with incomplete media. Red blood cells were eliminated by ACK lysis buffer and washed twice again. Cell numbers and viability were assessed by the trypan blue exclusion method. Lymphocytes were resuspended in RPMI

media containing 10 % fetal calf sera (FCS) and 2mM L-glutamine.

ELISPOT assays: B cells actively producing antibodies were enumerated utilizing an ELISPOT system as described (Verthelyi and Ansar Ahmed, 1996b). Briefly, 96 well-flat bottom plates (Costar, high binding ELISA plates were coated with acetone-killed *Brucella abortus* strain RB51 (2 μg/well in coating buffer), fraction S2 of *Brucella abortus* strain RB51 (5 μg/well in coating buffer), dsDNA (Sigma, St Louis, Mo; 70 μg/ml in PBS), cardiolipin (Sigma, St Louis, Mo; 50 μg/ml in ethanol), actin (Sigma, St Louis, Mo; 1 μg/ml in PBS), *L. monocytogenes* (2 μg/ml in coating buffer, or heavy chain specific goat anti mouse Ig (Southern Biotechnology Assoc., Birmingham, AL., 1 μg/ml in PBS). Plates were incubated for 48 hours under sterile conditions at 4°C, covered. Plates were then washed with PBS and blocked for 2 hours with 125 μl per well of PBS-2% BSA. Single cell suspensions (100 ul) from spleen were the plated at 5 to 0.2 x 10^6 cells per ml for B cells producing antibodies to cardiolipin, dsDNA, actin, *Brucella* or *Listeria*, and 0.2 to 1 x 10^5 cells per ml for B cells actively secreting IgG or IgM immunoglobulins. Cells were incubated for 6 hours at 37°C in 5% CO_2 incubators. Wells were thoroughly washed (5 times) with PBS and alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (1:3000, Southern Biotechnology, Birmingham, AL) in PBS 2% BSA were used as secondary antibodies. Five-bromo-4-chloro-3-indolyl phosphate (BCIP) at 2.3 mM in 2-amino-2-methyl-1-propanol buffer (AMP buffer) was mixed in 4:1 ratio with 3% agarose to be used as substrate.

Studies on apoptosis: The proportion of cells undergoing apoptosis was assessed utilizing acridine orange and ethidium bromide as previously described (McGahon et

al., 1995; Verthelyi and Ansar Ahmed, 1996b). Briefly cells are resuspended in media or PBS and 1 μl of dye mixture (100 μg/ml acridine orange + 100 μg/ml ethidium bromide) per 25 μl of cells are mixed gently and observed under UV light. At least 200 cells were counted per sample.

Statistical Analysis: Non-parametric ANOVA or Mann-Whitney tests were employed were employed to assess differences among treatment groups given the lack of normality of the distribution of the data.

RESULTS:

Estrogen induces the expression of IgG and IgM antibodies to cardiolipin, dsDNA and *Brucella abortus* strain RB51 in naive BALB/c female mice:

To assess whether nonautoimmune BALB/c mice would develop antibodies to self and hetero (Brucella) antigens after a short exposure (30 days) to estrogen, mice were treated with estrogen (n=20) or placebo (n=20) implants. At the time of removal of the implants their sera was tested for the presence of antibodies to a panel of auto and heteroantigens. As shown in **figure 8.1**, at the time of the removal of the implants, the mice treated with estrogen had significantly higher levels of IgM and IgG antibodies to cardiolipin, dsDNA and acetone-killed RB51 (**figure 8.1 a, b & c**). In addition, similarly to what we had previously reported (Verthelyi and Ansar Ahmed, 1996), estrogen induced the expression of antibodies to actin, ovalbumin, acetone-killed *listeria* in nonautoimmune BALB/c mice (data not shown).

Estrogen-treatment did not delay the clearance of Brucella:

The development of protection against *Brucella abortus* has been shown to require both humoral and cell mediated responses. The presence of antibodies is thought to be important to reduce the infective load, while cell-mediated immunity is thought to be responsible of the elimination of the infected cells. Treatment with estrogen has been show to enhance antibody production and to depressed cell mediated immunity (reviewed in Ansar Ahmed et al., 1985). To assess whether exposure to estrogen would delay the clearance of *Brucella abortus* strain RB51, we vaccinated 10 estrogen-treated and 10 placebo-treated mice with *Brucella abortus* strain RB51 in Freunds adjuvant. Estrogen- and placebo-treated mice that received saline-adjuvant served as controls. Previously reported virulence studies in BALB/c mice had shown that *B. abortus* strain RB51 is cleared within 4 to 5 weeks of inoculation (1×10^8 to 1×10^9 c.f.u.) (Schurig et al., 1991). After 5 weeks no live bacteria could be recovered from any of the treatment groups.

Estrogen treatment does not alter the humoral response to *Brucella abortus* strain RB51 vaccine:

As Shown in **figure 8.2**, the levels of IgG and IgM antibodies to RB51 at terminal bleeding (ten weeks after estrogen treatment) were significantly higher in vaccinated mice, regardless of whether they had received estrogen or placebo implants. The level of antibodies to RB51 in unvaccinated estrogen-treated mice, although higher than placebo-treated unvaccinated mice, did not reach that of mice that were vaccinated with RB51 ($p=0.027$).

Vaccination with RB51 did not limit the estrogen-induced expression of antibodies to auto- or hetero-antigens. Estrogen-treated mice had significantly higher levels of IgG antibodies to cardiolipin than controls. Similar increases were observed for antibodies to dsDNA, ovalbumin, actin and acetone-killed listeria (data not shown). IgM antibodies to ovalbumin were elicited by treatment with both, estrogen and RB51, their effect, however, was not additive. Interestingly, placebo-treated mice vaccinated with RB51 showed an increase in the levels of IgG antibodies to cardiolipin compared to those placebo-treated mice that were vaccinated with saline ($p < .001$). Similar trends were determined for IgM anticardiolipin antibodies. None of the groups had anticardiolipin antibodies of IgA isotype.

The anticardiolipin antibodies induced by estrogen treatment in BALB/c mice are of IgG1 and IgG2b subisotypes:

Mice treated with estrogen had increased IgG antibodies to cardiolipin. In previous studies we had shown that estrogen-induced IgG antibodies to cardiolipin in C57BL/6 mice were mainly of IgG2b and to a lesser extent IgG1 and IgG3 subisotypes. As shown in **figure 8.3** the antibodies to cardiolipin elicited by estrogen in BALB/c mice also were of IgG1 and IgG2b and to a lesser extent IgG3 subisotypes. Antibodies to cardiolipin evoked by vaccination with *Brucella abortus* strain RB51 belonged to IgG2a, IgG2b and IgG3 subisotypes. This indicates that the predominance of subisotypes IgG2b and IgG1 is determined by the treatment with estrogen, and not by the strain of mice utilized.

Mice treated with estrogen had increased numbers of activated immunoglobulin-producing cells in spleen, regardless of their subsequent vaccination with RB51.

At the cellular level, we had previously shown that estrogen induced the activation of splenic lymphocytes in nonautoimmune C57BL/6 mice, with increased number of immunoglobulin-producing cells as well as increased number of cells spontaneously producing antibodies to cardiolipin, dsDNA, actin and ovalbumin. Paired analysis of the number of immunoglobulin-producing cells of IgM and IgG isotype from spleen were significantly higher ($p=0.0028$) in mice treated with estrogen, regardless of whether they had been vaccinated with RB51. Among the placebo treated mice, however, there was a significant increase ($p=0.01$) in total IgM and total IgG in the RB51 vaccinated group compared to the controls **(figure 8.4a)**.

Cells spontaneously producing IgG antibodies to RB51 (acetone-killed whole cell or S2 fraction) were only detectable in mice vaccinated with RB51, regardless of estrogen treatment. Cells producing IgM antibodies to these same antigens, were significantly increased by estrogen treatment and by RB51 vaccination. The two treatments did not appear to have an additive effect **(Figure 8.4b)**.

Antibody forming cells of IgM isotype to all other antigens tested were increased in estrogen-treated BALB/c mice, regardless of whether they were vaccinated with RB51 as shown in **figure 8.4b**. Under normal circumstances lymphocytes spontaneously secreting IgG antibodies to the antigens tested are nearly undetectable. Estrogen treatment, however, induced the activation of cells producing IgG antibodies to cardiolipin, dsDNA, actin and listeria (data not shown). Lymphocytes from estrogen-

treated mice that had been vaccinated had slightly lower numbers of active IgG antibody forming cells to cardiolipin, and dsDNA.

Exposure to estrogen appears to increase apoptosis in freshly isolated spleen cells:

Apoptosis was assessed by staining fresh cells with acridine orange and ethidium bromide. As shown in **figure 8.5**, the number of cells undergoing apoptosis was minimal in the PS group, where 3.6% were in early apoptosis and no apoptotic cells were found. Mice treated with estrogen had a higher proportion (14%) of spleen lymphocytes undergoing apoptosis (10.2% in early apoptosis and 3.8% apoptotic). Vaccination with RB51 also induced cell death in spleen lymphocytes (2.6% in early apoptosis, and 3.4% apoptotic). Mice that were exposed to both estrogen and RB51 (EB), had the highest percentage of cells undergoing apoptosis (19.82%; 12.1% early apoptotic and 7.7% late apoptosis.) All groups had approximately 11% of cells dying by necrosis.

DISCUSSION:

The objectives of the present study were twofold: First, to assess whether estrogen treatment would have a similar effect on the immune system of a different nonautoimmune strain of mice. Second, to determine whether exposure to estrogen would affect the immune response to subsequent vaccination with an infectious agent. Since treatment with estrogen elicited the spontaneous expression of antibodies to B. abortus strain RB51 whole organisms, we utilized this strain to challenge the mice (Schurig et al., 1991).

Brucella abortus is a facultative intracellular pathogen that infects humans and a variety of domestic animals. The clinical manifestations of brucellosis can include arthritis, endocarditis and meningitis in humans, and spontaneous abortions in cattle. Protective immunity against *Brucella abortus* in mice has been shown to be mediated by humoral as well as cell mediated immunity (Winter et al., 1989). Antibodies to the O-chain polysaccharide of *Brucella abortus* LPS have been shown to confer some protection in BALB/c mice in passive transfer experiments. Transfer of either $CD4^+$ or $CD8^+$ T cells from Brucella-infected mice to naive mice have also been shown to protect against the disease, indicating that cellular immunity is required for the elimination of this facultative intracellular pathogen. In this study we have used this pathogen to evaluate the response of estrogen treated mice to infection.

We report that nonautoimmune BALB/c mice expressed in sera autoantibodies to all antigens tested, including cardiolipin, dsDNA, actin, ovalbumin, as well as acetone-killed *B. abortus* strain RB51 and *L. monocytogenes*. At a cellular level, we show that estrogen elicited a generalized increase in the number of immunoglobulin forming cells. Specific antibody-forming cells of IgG isotype directed to the antigens tested under normal circumstance are barely detectable by ELISPOT (less than 0.2 per 1 x 10^6 cells). However, in mice treated with estrogen, cells spontaneously secreting IgG antibodies to these antigens were readily evident (>1 per 10^6 cells). Further, the number of IgM antibody producing cells to cardiolipin, dsDNA, actin, *B. abortus* and *L. monocytogenes* was significantly increased. These findings suggest that estrogens elicit a polyclonal B cell activation of splenic lymphocytes. In accordance with our previous reports (Verthelyi and Ansar Ahmed, 1996), our preliminary studies show that spleen lymphocytes from estrogen-treated mice vaccinated with saline cultured in

complete media proliferated even in the absence of stimuli (data not shown). Upon stimulation, lymphocytes may be activated to undergo differentiation and proliferation, or may be driven to undergo apoptosis. In estrogen-treated nonautoimmune BALB/c mice, freshly isolated spleen cells had higher numbers of cells undergoing apoptosis that the controls. This result, together with the increase in spontaneous proliferation, reinforces the notion that estrogen has a modulatory effect on the progression of lymphocytes through their cell cycle.

To assess how estrogen would modulate the immune response to infection, we utilized *B.abortus* strain RB51. We utilized this strain to challenge the mice since: (i) Treatment with estrogen elicited the spontaneous expression of antibodies to B. abortus strain RB51 (Verthelyi and Ansar Ahmed, 1996b). (ii) Infection with *B. abortus* has been shown to induce Th1 type cytokines (Oliveira et al.,1996) and (iii) Clearance of *B. abortus* requires the presence of helper and cytotoxic T cells (Araya et al., 1989; Araya and Winter A.J., 1990), which have been reported to be depressed in mice treated with estrogen (reviewed in Ansar Ahmed and Talal, 1990).

Clearance of *B. abortus* strain RB51 was not delayed in mice treated with estrogen and vaccinated with *B. abortus* (EB) compared to those treated with placebo (PB) or previous reports (Schurig et al., 1991). Further, the level of antibodies to *B. abortus* elicited in sera by the vaccine were not affected by the prior exposure to estrogen. It is interesting to note that although IgG and IgM antibodies to RB51 were higher in placebo-treated mice vaccinated with RB51 (PB) than in estrogen-treated mice that did not receive the vaccine (ES), the number of activated IgM antibody-producing cells in spleen was higher in ES than PB mice. A possible explanation for this discrepancy is

that while estrogen has been shown to activate splenic antibody-forming cells, antibody-forming cells elicited by the RB51 vaccine may home to a different tissue (eg. bone marrow). Also, the ELISPOT assay showed that EB had slightly reduced number of IgM antibody-forming cells than ES mice (though higher than PS mice).

In vivo exposure to both, estrogen and RB51, increased the number of fresh spleen cells undergoing apoptosis. The effect appeared to be additive, since spleen lymphocytes from mice that were exposed to both, estrogen and RB51 (EB), had twice as many cells dying. The uniform number of cells dying by necrosis further validated these results.

An interesting observation made in these experiments is that vaccination with attenuated, rough strain RB51 elicits the expression of IgG antibodies to cardiolipin. Induction of antibodies to cardiolipin by infectious agents has been reported previously in bacteria (syphilis), virus (AIDS, mumps, chicken pox) and rickettsias (Lyme disease). Interestingly, *B.abortus* strain 2308, the highly virulent and smooth parent strain of RB51 does not induce the expression of these antibodies **(appendix a).** These findings pose interesting questions on the possible role of antibodies to cardiolipin in brucellosis that are being actively pursued by our laboratory. The antibodies to cardiolipin elicited by *B. abortus* belonged to different subisotypes (IgG2a, IgG2b and IgG3) than those induced by estrogen treatment (IgG2b, IgG1 and to lesser extent IgG3). Gamma interferon, a Th1 cytokine, thought to be induced by exposure to *B. abortus* has previously been shown to induce a switch to IgG2a subisotype; TGF-β has been postulated to promote the switch to IgG2b; and IL-4 is thought to mediate the switch to IgG1. The binding to cardiolipin coated plates was

not enhanced by addition of human β2-glycoprotein I in either group (data not shown).

Together our study shows that exposure to estrogen modulates the immune system of nonautoimmune mice by inducing the expression of a variety of antibodies, and activating the immunoglobulin producing cells in spleen. These effects, however, had a limited effect on the immune response to *B.abortus*.

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Table 1: Treatment groups

TREATMENT GROUP	ESTROGEN	*Brucella abortus* strain RB51
PS	placebo	saline
PB	placebo	RB51
ES	17β-estradiol	saline
EB	17β-estradiol	RB51

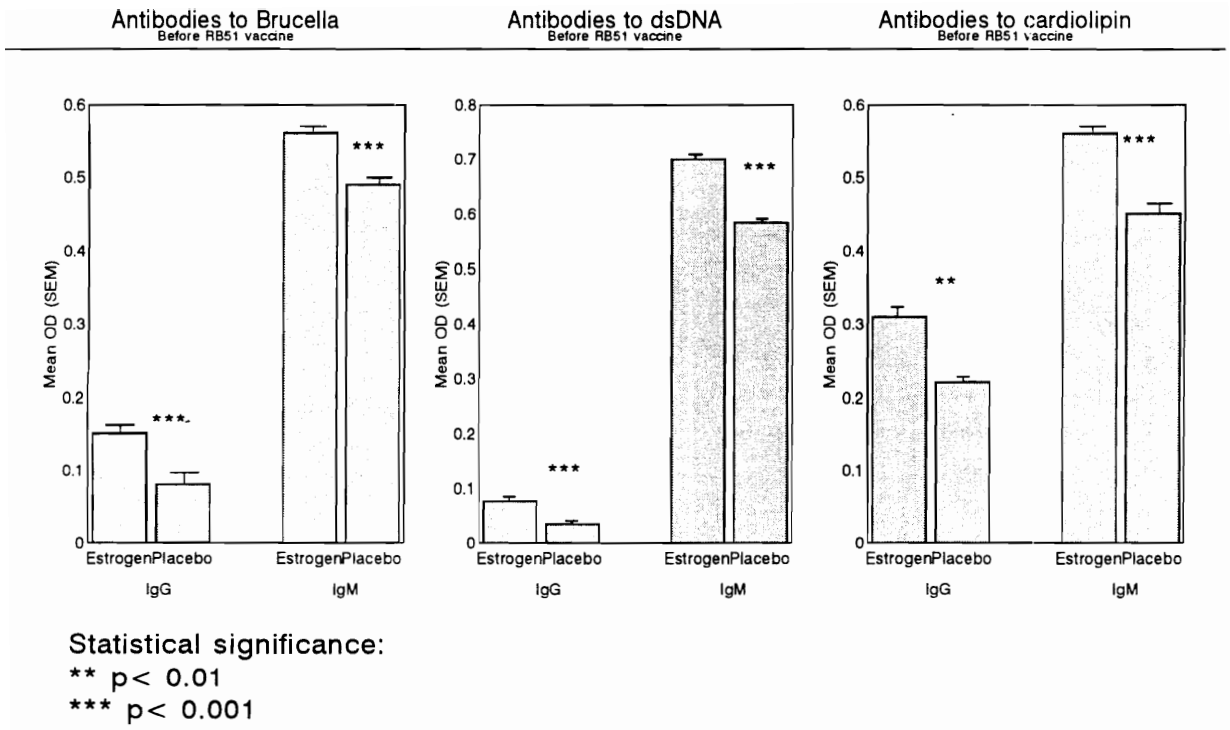


Figure 8.1: Sera of young (2-3 month-old) BALB/c female mice treated with 17β-estradiol (n=20) or placebo implants (n=20) were tested for IgG antibodies to *B. abortus,* cardiolipin and dsDNA by ELISA. Statistical analysis was performed using Mann Whitney non-parametric test.

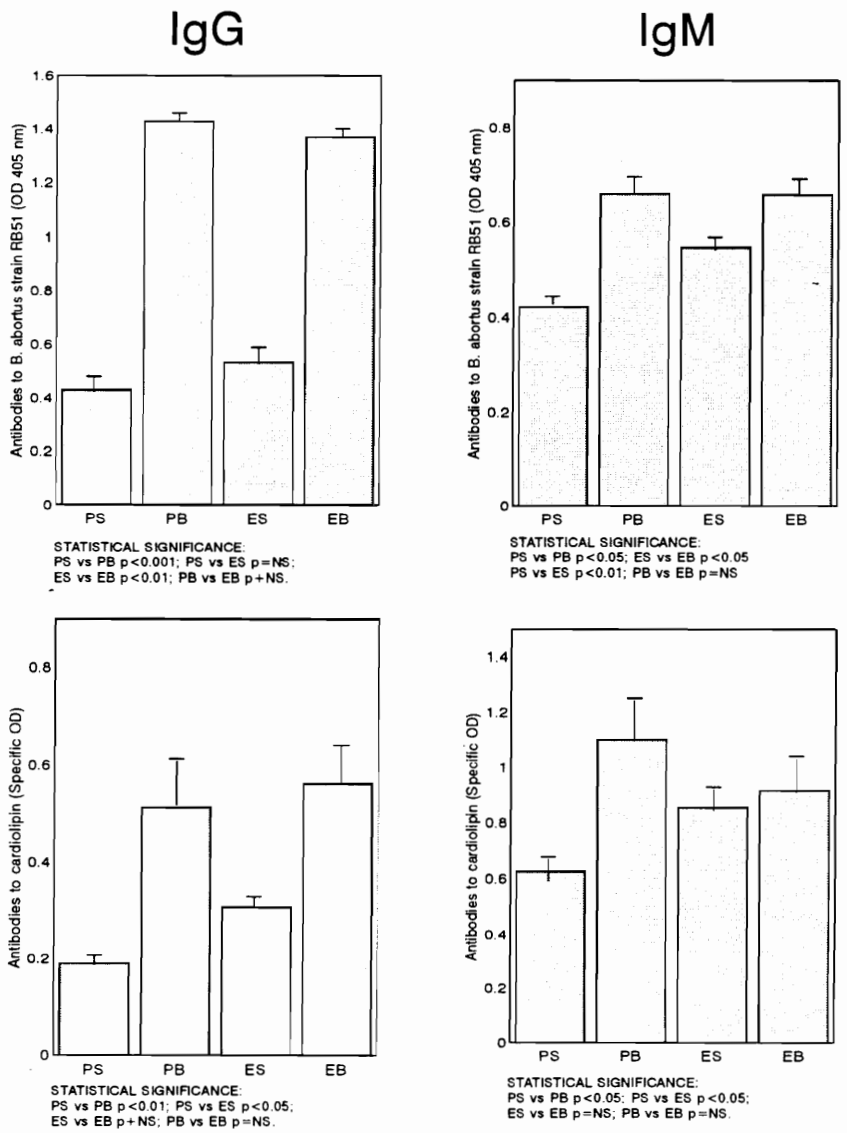
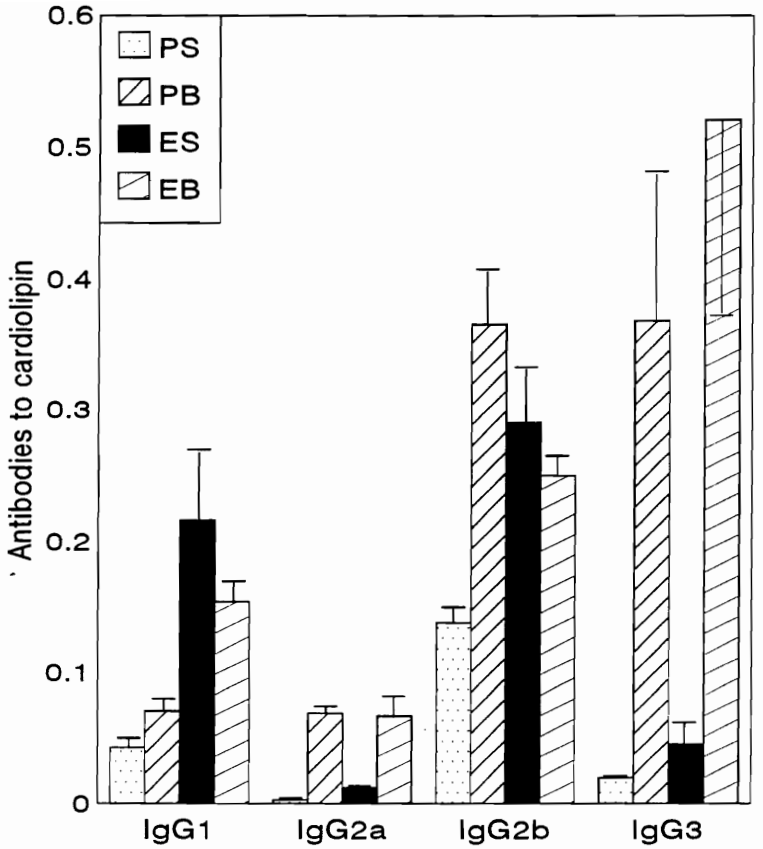


Figure 8.2: The level of antibodies to *B.abortus* strain RB51 and cardiolipin of IgG and IgM isotype were determined in sera of BALB/c female mice of the four treatment groups (n=10 per group). Statistical analysis was performed utilizing Kruskal Wallis nonparametric test.



Statistical significance:

	PS vs PB	PS vs ES	ES vs EB	PB vs EB
IgG1	NS	p<0.01	NS	p<0.05
IgG2a	p<0.001	NS	p<0.01	NS
IgG2b	p<0.001	p<0.05	NS	NS
IgG3	p<0.001	NS1	p<0.001	NS

Figure 8.3: Subisotype distribution of the antibodies to cardiolipin induced by exposure to estrogen (ES), to B.abortus strain RB51 (PB), and to both (EB). Mice treated with placebos and vaccinated with saline (PS) served as controls.

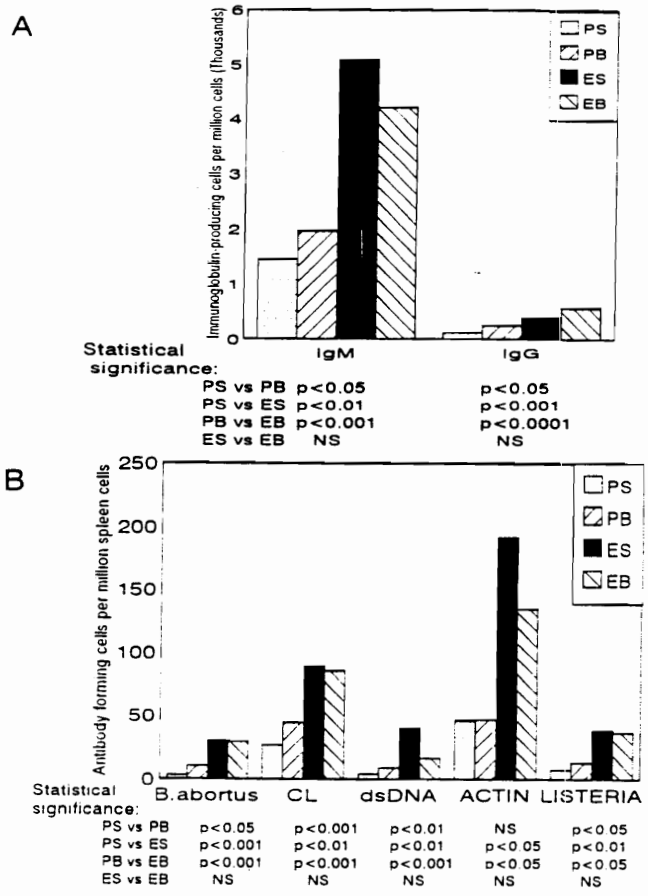


Figure 8.4: (a) Total immunoglobulin-producing cells of IgG and IgM isotype were quantitated using an ELISPOT assay. Note that the number of lymphocytes spontaneously secreting immunoglobulins from estrogen treated mice [ES (n=10), and EB (n=9)) is significantly higher than that of mice that received placebo implants [PS (n=10) or PB (n=9)). (b) Shows the number of spleen cells spontaneously secreting antibodies of IgM isotype to specific auto- and hetero-antigens.

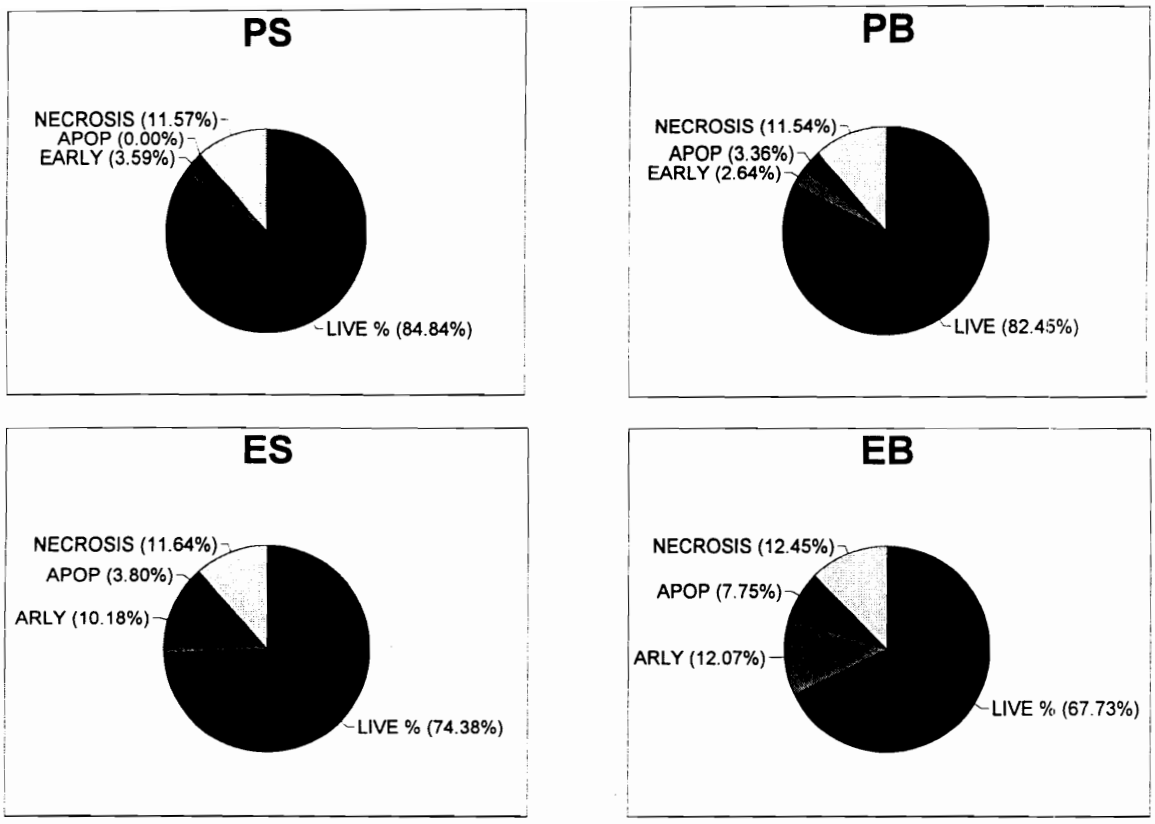
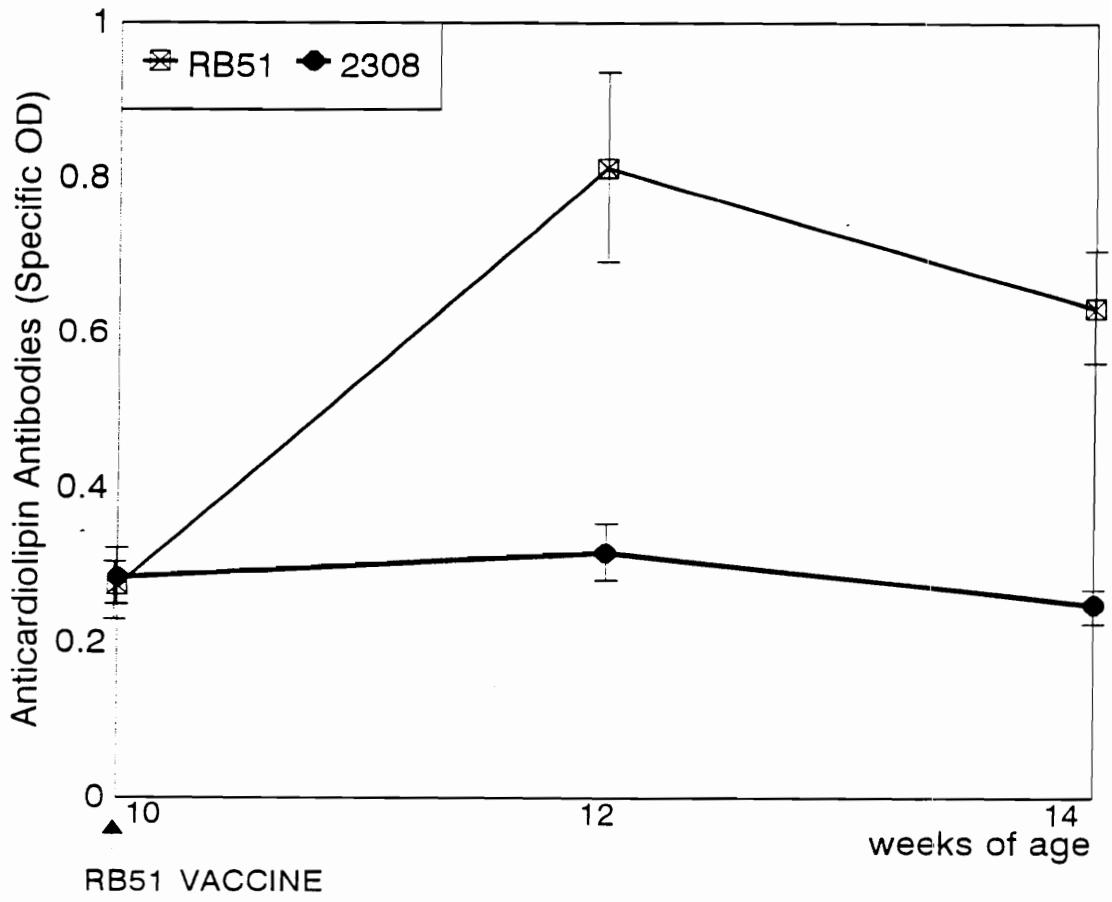


Figure 8.5: Proportion of freshly isolated spleen cells undergoing cell death by necrosis or apoptosis as appraised by acridine orange and ethidium bromide technique

Appendix A



Antibodies to cardiolipin are elicited in mice vaccinated with *Brucella abortus* strain RB51 but not by parent strain 2308:

Strain RB51 is a rough and attenuated mutant of *Brucella abortus* strain 2308. Since expression of antibodies to cardiolipin have been associated with abortions, a common feature of infection with *B.abortus* strain 2308, we assessed whether vaccination with this strain would elicit antibodies to cardiolipin. As shown in appendix A vaccination with strain RB51, which does not induce abortions, elicited IgG antibodies to cardiolipin; while infection with the abortifacient strain 2308 did not.

CHAPTER 9

DISCUSSION:

This chapter compiles all our findings and attempts to arrive at cohesive conclusions. I have speculated on possible mechanisms by which estrogen would promote B cell functions. It is acknowledged that other possibilities exist.

Advantages and limitation of using C57BL/6 mice as a model:

The majority of the studies were performed on C57BL/6J mice which are currently considered as a non-autoimmune strain (Ansar Ahmed, 1994). This strain, however, has been shown to develop Sjögren syndrome-like lesions in old age (Hayashi et al., 1989). Also, C57BL/6 mice constitute part of the genetic background of mice that spontaneously develop autoimmune disease (BXSB/Mp mice; Ansar Ahmed, 1994). Since the development of autoimmune diseases is multifactorial (genetic, environmental, hormonal, infectious factors, etc.), C57BL/6J mice was thought to represent a good model to study the influence of sex steroids on healthy individuals who may have some predisposing factors for autoimmune disease in their background. We have shown that C57BL/6 mice treated with estrogen develop a variety of auto- and hetero-antibodies, manifest signs of B cell hyperactivity, show defects in susceptibility to apoptosis, and excessive proliferation in the absence of stimulation. These qualities appear to make estrogen-treated C57BL/6 mice a potential animal model for studying autoimmunity and possibly autoimmune diseases. The possible relevance of this model was underscored by clinical findings that women who underwent *in vitro* fertilization treatment, which leads to

increased levels of estrogen, developed an SLE-like disease (Yron et al., 1992; Ben-Chetrit and Ben-Chetrit, 1994).

It must be noted, however, that (i) the C57BL/6 mice represent an inbread population of mice, and (2), that the pathogenic role of the autoantibodies elicited by treatment of C57BL/6 mice with estrogen has not been established. Further, the lack of requirement for β2-glycoprotein I for binding of estrogen-induced antibodies to cardiolipin resembles that of the antibodies to cardiolipin elicited by infection (Nevertheless, we showed similar β2-requirements in MRL/lpr and BXSB mice, which are established models for SLE). These mice were not thrombocytopenic (chapter 3). The question of whether estrogen-treated normal mice can drive from autoimmunity state (autoantibodies) to autoimmune diseases needs to be addressed. Further, the precise immunopathogenic effect of estrogen in this murine model warrants further investigation. Nevertheless, estrogen-treated C57BL/6 mice constitute a good model for studying the immune consequences of sex hormonal treatment.

The effects of estrogen-treatment on B cell functions:

We have found that unmanipulated nonautoimmune C57BL/6 mice, particularly females have low levels of IgM and IgG antibodies to cardiolipin and dsDNA (Chapters 3 & 4). Orchiectomy or the administration of male hormones (DHT) did not alter the incidence or level of these antibodies, but treatment with estrogen had a strong autoantibody-promoting effect both in male and female mice (Chapters 3 & 4). Similar effects were observed in female BALB/c mice treated with estrogen indicating that the effects of estrogen as an inducer of autoantibodies to cardiolipin or dsDNA are not restricted to

C57BL/6 mice (Chapter 3 and 8). Previous studies on the immunomodulatory effects of estrogen reported augmented level of antibodies to many exogenous antigens in normal mice and rats (Meyers and Petersen, 1985; reviewed in Ansar Ahmed et al.,1985 ; Schuurs and Verheul, 1990; Ansar Ahmed and Talal, 1990); and to a variety of autoantigens in autoimmune mice (Roubinan et al., 1978; Steinberg et al., 1979; Brick et al., 1985). Further, estrogen treatment had been shown to induce IgM autoantibodies to bromelin-treated red blood cells in C57BL/6 mice, presumably secreted by B1 cells (Ansar Ahmed et al., 1989). However, these are the first reports of estrogen-induced autoantibodies of IgG isotype in nonautoimmune mice (Chapter 3 and chapter 4). It must be emphasized that other than hormonal treatment these mice were not injected with DNA, cardiolipin or any other antigen or preparation from infectious agents.

We found that autoantibodies to cardiolipin and dsDNA belonged to IgM and IgG but not IgA isotypes. The subisotype distribution showed that these estrogen-induced autoantibodies, both in C57BL and in BALB/c mice, were mainly of IgG2b subisotype. It is intriguing to speculate the cytokines involved in IgG2b isotype switch. Transforming growth factor-β has been shown to promote the switch to IgG2b (McIntyre et al., 1993). However, TGF-β has also been reported to promote isotype switching to IgA (reviewed in Massague, 1990), an isotype we do not find to be increased in our estrogen-treated mice (Chapters 4 and 5). The marked predominance of IgG2b isotype, together with data in the literature for estrogen-induced TGF-β in bone (Finkelman et al., 1992) and rat gonads (Dorrington et al., 1993), as well as reports on increased levels of TGF-β in SLE patients (del Giudice and Crow, 1993) prompted us to asses the levels of TGF-β in the sera of mice treated with estrogen. We found that male and female mice treated with estrogen had reduced levels of TGF-β_1 in sera compared to controls (data

not shown). It is possible, that despite reduced levels of TGF-β in sera, mice treated with estrogen could have increased levels of TGF-β at (other) specific anatomical sites. The reduction in TGF-β in sera, could be partially responsible for the increase in IgG and IgM, since TGF-β is known to reduce their synthesis and secretion in response to T cell-dependent antigens (reviewed in Massague, 1990). The possibility that TGF-β-like molecules, akin to those found in synovial fluid of RA patients (Abedi-Valugerdi et al., 1993), may be involved in the isotype switch to IgG2b needs to be examined.

The degree of crossreactivity of antibodies to cardiolipin with other selfantigens varied. Antibodies to cardiolipin crossreacted extensively with other anionic phospholipids, but showed little crossreactivity with zwitterionic phospholipids (Ansar Ahmed et al., 1993), dsDNA or bDNA (Chapter 5).

In our studies we have shown that nonautoimmune mice exposed to estrogen develop not only IgG antibodies to dsDNA, cardiolipin and several anionic membrane phospholipids (chapters 3, 4, and 5) but to a variety of heteroantigens including heterologous proteins such as ovoalbumin, lysozyme and infectious antigens such as *B. abortus, L. monocytogenes* or *A. pleuropneumoniae* as well (Chapters 5, 6 and 8). There was minimal crossreactivity of antibodies to cardiolipin with heteroantigens like ovoalbumin (Chapter 5). The spontaneous secretion of antibodies to *B abortus* strain RB51 did not delay the clearance of the bacteria. Further, the level of antibodies to *B.abortus* elicited by vaccination with RB51 was unaffected, indicating that, despite the dysregulation of the B cell function elicited by estrogen treatment, the mice were able to respond adequately to an infectious challenge.

The estrogen-mediated increase in autoantibody production was also confirmed at the cellular level. In spleen and bone marrow, estrogen induced an increase in the number of IgG and IgM immunoglobulin producing cells. The antibody per cell yield of plasma cells was also enhanced in these mice as evidenced by the increase in the area and density of the spots generated by individual cells in the ELISPOT assay. However, although estrogen induced an increase in the number of plasma cells in spleen (chapter 6), and a significant increase in the number of B cells actively secreting antibodies to auto- and hetero-antigens, the proportion of activated plasma cells devoted to various antigens studied was similar in mice treated with estrogen and placebo controls. These results suggest that estrogen may induce a polyclonal B cell stimulation. It must be noted, however, that only a handful of antigens were tested, and that to confirm this aspect, studies should be extended to include a larger panel of antigens. There are several ways by which estrogen could induce an increase in the number antibody-producing cells in spleen. Estrogen could induce an increase in the number of mature resting B cells that become plasma cells. This possibility is supported by our findings (Chapter 7) that *ex vivo* lymphocytes from mice treated with estrogen have increased numbers of B cells in the S and M/G2 phases of cell cycle, as well as increased spontaneous proliferation *in vitro,* even in the absence of evident stimulation. Alternatively, estrogen could prolong the plasma cell viability by altering the susceptibility of plasma cells to die. For example, estrogen could induce B cells to have increased levels of Bcl/2, or reduced expression of Fas on the activated B cell's surface **(figure 9.1)**. And indeed, in chapter 7, we report that estrogen does render spleen B cells more resistant to death by apoptosis in the presence and absence of stimulation.

Autoreactive B cells could arise at three main points during the B cell life span. These

include: (i) during B cell development; (ii) during affinity maturation of B cells to produce high affinity plasma and memory cells, and (iii) immunoregulatory defects leading to continued immune response, even after the stimulating antigen has been eliminated (**Figure 9.2**). **Figure 9.1** represents a selection of possible mechanism by which estrogen could elicit the changes in the B cell functions. Estrogen has been shown to act on bone and bone marrow to induce osteopetrosis and a reduction in bone marrow cellular mass (Seaman et al.,1978). Bone marrow, not only is an important hematopoietic organ in the mice, but also acts as an important secondary lymphoid organ. Early in life, bone marrow contains predominately immature B cells. Although B cells continue to be produced in bone marrow throughout life, with age, increasing numbers of antibody-forming cells have been shown to accumulate in bone marrow (reviewed in Benner et al., 1981; Nuñez et al., 1996). Bone marrow is considered to be a major source of serum immunoglobulins of IgM, IgG and IgA isotypes (reviewed in Benner et al., 1981). Treatment with estrogen has been shown to induce the death of pre-B cells at the IL-7 sensitive stage (Kincade, 1994; Masuzawa, 1993). It is possible that precursors for autoreactive clones are more resistant to estrogen-mediated death than normal B cells, and therefore autoreactive B cells would expand preferentially. Alternatively, the depletion of bone marrow cellularity may draw lymphopoiesis to other sites. We have noticed signs of extramedullary hematopoiesis in liver and spleen. It is possible that the selection processes (positive and negative), in these alternative sites of hematopoiesis are not as stringent, or as efficient as those present in bone marrow thereby allowing autoreactive clones to develop.

Alteration of bone marrow integrity could also disrupt the safeguarding mechanisms during somatic mutation of activated B cells. As mentioned above, bone marrow is also

an important site for mature B cells to evolve into memory or plasma cells. The increased local cellular debris due to estrogen-induced reduction in bone marrow mass, and the increased bone turnover, may expose B cells to self antigens that ordinarily would be sequestered. In our studies on B cell function we have shown that B cells from mice treated with estrogen are resistant to death by apoptosis even in the absence of stimulation. It is possible that the decreased susceptibility to apoptosis is mediated by estrogen-induced higher basal levels of Bcl/2 or other compounds that block or retard apoptosis. Under normal circumstances, as explained in the literature review, B cells undergo somatic mutations and cells that do not have high affinity for the antigen are not rescued from death by apoptosis. Increased levels of Bcl/2 or Bcl/x would prevent somatically mutated self reactive B cells from death, and further, the presence of cellular debris could induce the preferential expansion of autoreactive clones. This mechanism would explain the expansion of B cells producing antibodies to cardiolipin and dsDNA that we found in bone marrow lymphocytes from mice treated with estrogen. This thought is compatible with findings in the literature, that overexpression of bcl/2 has been shown to lead to the expression of autoreactive clones (Strasser et al., 1991).

Lastly, defective elimination of plasma cells may result from reduced expression of fas antigen on the cell surface of activated cells. Defects in apoptosis of activated lymphocytes has been shown to be an important pathogenic mechanism in lpr and gld mice which are animal models for systemic lupus erythematosus (SLE) Gillette-Ferguson and Sigman, 1994; Rose et al., 1994).

It must be noted, however, that *in vivo,* spleen lymphocytes from nonautoimmune mice treated with estrogen showed increased numbers of B cells dying by apoptosis. The

finding of cells dying by apoptosis in B cells freshly isolated from spleen agrees with previous reports (Illera, 1993). The partial disagreement between the increased levels of apoptosis in vivo and the resistance of B cells to undergo apoptosis *in vitro,* suggest that *in vivo* B cells are driven to death by signals from the surrounding stromal cells. Estrogen-induced death of pre-B cells in bone marrow has been reported in bone marrow, where estrogen is thought to act on stromal cells which then produce an as yet unidentified factor that leads pre-B cells to death (Smithson et al.,1995).

The mechanisms of action of estrogen are highly complex. Estrogen is a pleiotropic hormone that acts on multiple targets including the CNS, hypothalamo-hypophyseal-thymic axis, bone, reproductive organs, and monocytes and macrophages of the immune system among others. Conceivably, estrogen could influence B cell function by acting indirectly via other target tissues (for example, estrogen has also been proposed to modulate the immune function by acting through the hypothalamo-hypophyseal-thymic axis to alter the neuro-immuno-endocrine regulatory circuits (Grossman, 1985)), or via other cells in the immune system (Estrogen receptors have been shown in macrophages (Cutolo et al., 1993) and $CD8^+$ T cells (Cohen et al.,1983). To date, the presence of estrogen receptors on B cells has not been conclusively determined. Delineating these mechanisms is a formidable task, therefore these studies focused on understanding the estrogen-induced alterations of the B cell functions (regardless of the direct or indirect effect).

It was the purpose of this work to determine whether estrogen modulated the B cell function. Together, these results indicate that induction of modest increases in the level of estradiol *in vivo* lead to long lasting B cell hyperactivity and production of

autoantibodies. Understanding the effects of estrogen on the immune system of normal individuals is of particular importance since women around the world take estrogen in the form of containing oral contraceptives or to alleviate the symptoms of menopause, some times for many years at a time. The immunological consequences of administration of estrogen for prolonged periods such as those occurring in estrogen replacement in the postmenopause need to be investigated. Last but not least, a growing number of pesticides, insecticides, and phytoestrogens have being found to have hormone disrupting effects with reproductive and immune consequences on a range of wild species. It is hoped that these studies will alert physicians about the immunological consequences of exposure to estrogen.

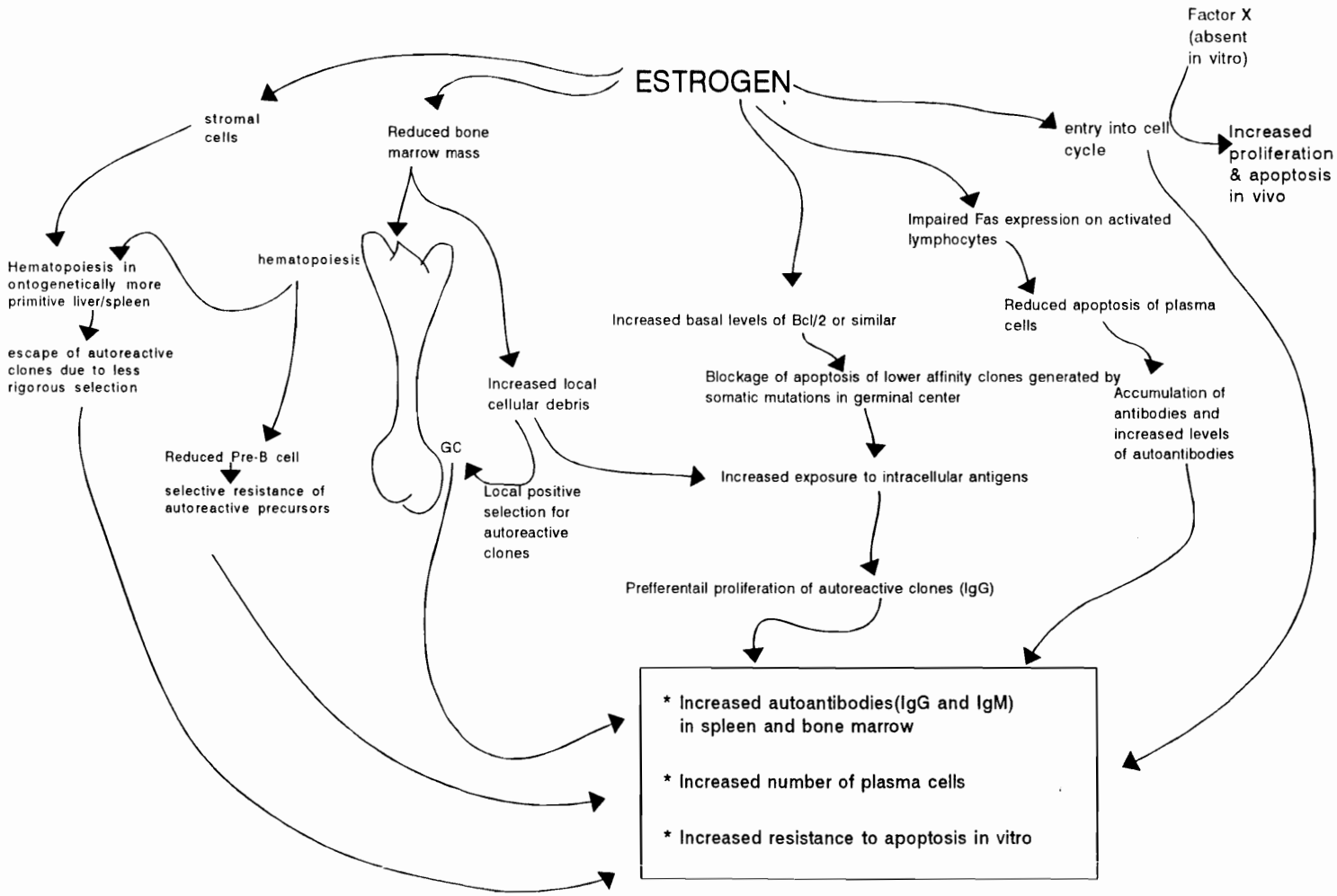
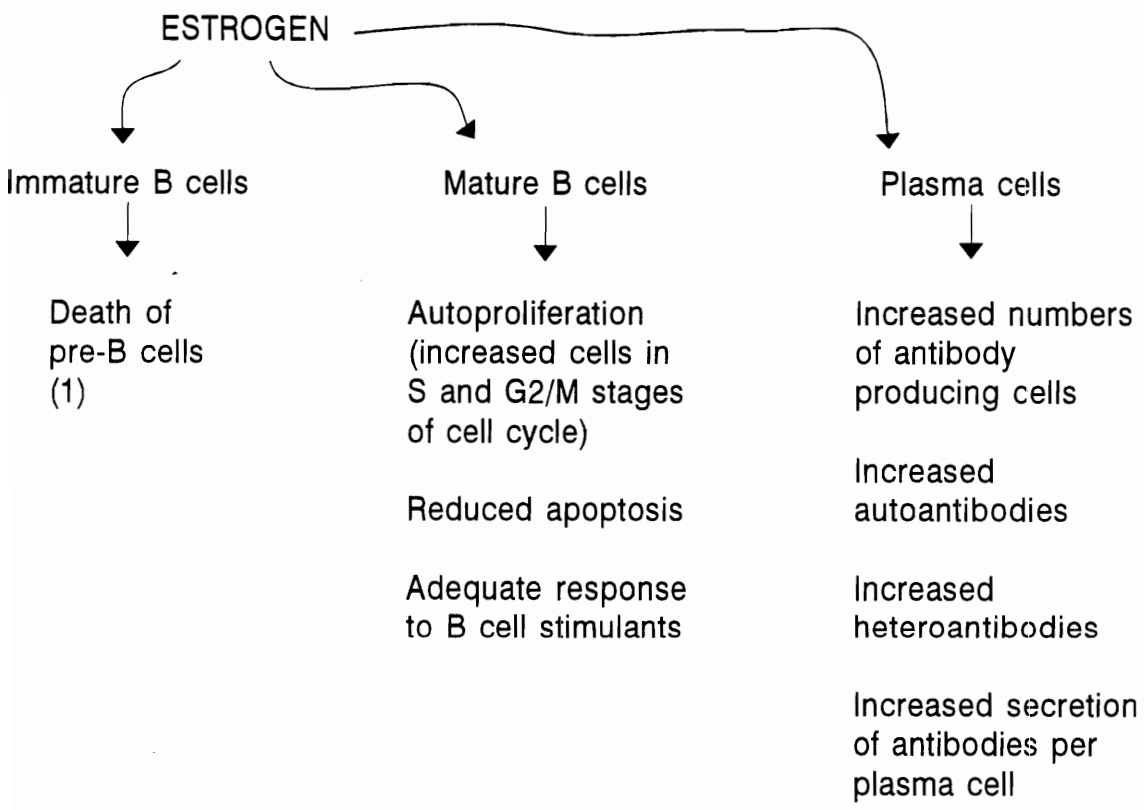


Figure 9.1: Possible mechanisms by which estrogen could induce the expression of autoantibodies

Estrogen can modulate B cell function at different stages.



CHAPTER 10

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VITA

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She is currently married to Eduardo Romano and has a daughter, Ari.