ALTERATION OF MURINE BONE MARROW B-CELL DEVELOPMENT AND FUNCTION BY PHYSIOLOGICAL CONCENTRATIONS OF GLUCOCORTICOIDS: A ROLE FOR PROGRAMMED CELL DEATH

By

Beth Anne Garvy

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Departments of Biochemistry and Physical Education and
Exercise Science

1991

ABSTRACT

ALTERATION OF MURINE BONE MARROW B-CELL DEVELOPMENT AND FUNCTION BY PHYSIOLOGICAL CONCENTRATIONS OF GLUCOCORTICOIDS: A ROLE FOR PROGRAMMED CELL DEATH

By

Beth Anne Garvy

Elevated plasma glucocorticoids generated by chronic stresses were known to cause lymphopenia and thymic atrophy. What effects such steroids might have on B-cell lymphopoiesis were unknown. Data presented herein demonstrate that physiological levels of glucocorticoids dramatically reduce the capacity of murine bone marrow to produce B-cells and that the depletion of such cells can be via induction of apoptosis. $B220^+$, IgM^+ and IgD^+ cells cultured with glucocorticoids formed a distinct peak to the left of G_0/G_1 in the hypodiploid region previously termed A_0, as determined by flow cytometric cell cycle analysis. Appearance of cells in the A_0 region correlated with internucleosomal DNA fragmentation and increased cell density, characteristics of apoptosis. Glucocorticoid-induced aptotosis of B-lineage cells was found to be dose-dependent and inhibitible by the glucocorticoid receptor antagonist RU38486. Further, bone marrow $B220^+$ cells were found to have similar numbers of GC receptors as thymocytes. To determine if glucocorticoids manifested

similar effects on bone marrow *in vivo*, corticosterone pellets were implanted subcutaneously which chronically elevated plasma GC analogous to the level observed during stress (30-100 $\mu g/dl$). Flow cytometric analysis of bone marrow B-lineage cells at day 5 indicated a depletion of $B220^{+}sIgM^{-}$ pre-B cells and $sIgM^{+}sIgD^{-}$ immature B-cells. However, a population of $B220^{bright}IgM^{+}IgD^{bright}$ cells increased two-fold and responded normally to antigenic challenge. Two-color cell cycle analysis indicated that the proportion of large cycling $B220^{+}$ cells in S phase had declined sharply. A distinct population of cells appeared in the A_o region between 6 and 36 hours after pellet implantation. Finally, prednisolone, a widely used anti-inflammatory glucocorticoid caused a three-fold reduction in bone marrow pre-B-cells when present in plasma at nanogram levels. Though the pattern of effects of prednisolone on B-lineage cells was somewhat different from that of corticosterone, it is clearly a potent suppressor of lymphopoiesis. Collectively, the data show that glucocorticoids at physiological levels have a profound effect on the development of B-cells, both *in vivo* and *in vitro* and that these steroids can readily induce apoptosis in developing B-cells.

Dedicated to:

Maureen, Daniel, Rachel, Brittany, Carly, and Chad

Commit your work to the Lord,

and your plans will be established.

Proverbs 16:3

ACKNOWLEDGMENTS

Special thanks to Dr. Pamela J. Fraker who helped me grow not only as a scientist but also in character. I am also thankful to Dr. William Heusner who always made the time to talk to me. I gratefully acknowledge the members of my guidance committee: Dr. David McConnell, Dr. Carol Rodgers, Dr. William Smith, and Dr. William Wells, who asked tough questions and gave valuable insight into the data presented in this dissertation. Thanks to Dr. Kathryn Brooks, Dr. Richard Schwartz who critically read my manuscripts and offered valuable suggestions. I am indebted to Dr. Michael Denison for generously giving up his time and materials to assist in teaching me various methods for glucocorticoid receptor analysis. I also am indebted to Vonnie Vander Ploeg in Dr. Clifford Welcsh's lab for teaching us a simple and inexpensive method of *in vivo* steroid delivery. Thanks to the past and present members of the Fraker lab: Joan Cook-Mills, Andrea Cress, Joe Gibbons, Lori Kurth, Sue Leak, Gerry Morford, and Lorri Morford for their friendship and support. I am particularly grateful to my collaborators: Dr. Louis King, Bill Telford, and Bryan Voetberg for their hard work, dedication, and friendship. I am greatly appreciative to the biochemistry and exercise physiology faculty and staff who were always willing to lend a helping hand. Thanks to Jack and Mary Beth Miller for a place to get away, and to Katy Read, my golfing buddy. Finally, I forever am indebted to my parents, Andy Garvy and Norma Garvy, who taught me to work at everything I do with my best effort. *Soli Deo Gloria*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

BM	bone marrow
cμ	cytoplasmic μ
CS	corticosterone
DX	dexamethasone
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GC	glucocorticoid
GcR	glucocorticoid receptor
GRE	glucocorticoid response element
HC	cortisol (hydrocortisone)
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
PD	prednisolone
PE	phycoerythrin
PFC	plaque forming cell
PI	propidium iodide
TdT	terminal deoxytransferase
TNP	trinitrophenol

Chapter 1

Literature Review

INTRODUCTION

Physiological stresses such as trauma, surgery, malnutrition, infection, burns, and exercise have been associated with a 2-5 fold increase in circulating cortisol (or corticosterone in mice) levels from concentrations of 5-20 μg/dl plasma to as high as 100 μg/dl (Di Padova *et al.*, 1991; Smith *et al.*, 1981; Alleyne & Young, 1967; DePasquale-Jardieu & Fraker, 1979; DePasquale-Jardieu & Fraker, 1980; Besedovsky *et al.*, 1975; Shek & Sabiston, 1983; Kagan *et al.*, 1989; Houmard *et al.*, 1990). Increased incidence of secondary infections possibly due to lymphopenia also are associated with these stresses (O'Mahony *et al.*, 1985; Kagan *et al.*, 1989; DePasquale-Jardieu & Fraker, 1980; Volenec *et al.*, 1979). The known immunosuppressive effects of glucocorticoids (GC) have raised the question of whether the lymphopenia associated with chronic stress is a result of the elevated GC levels. This is a particularly compelling question since GC have been shown to cause atrophy of the thymus, the site of T-lymphocyte maturation (Weissman, 1973; Screpanti *et al.*, 1989). Unfortunately most of what is known about the immunosuppressive properties of GC was determined after administration of large or acute doses of synthetic GC with comparatively high potencies. Additionally, most studies dealt with the effects of GC on peripheral lymphocytes or thymocytes and ignored possible effects on the immature developing B-cells found in the mammalian bone marrow (BM).

Using zinc deficiency as a model for malnutrition, our laboratory generated data which indicated that plasma corticosterone (CS) levels reached concentrations as high as 100 $\mu g/dl$ in zinc deficient mice (DePasquale-Jardieu & Fraker, 1979). While the response of zinc deficient mice to antigenic challenge was reduced in zinc deficient mice, the reduction in intensity of the response was related to reduced numbers of lymphocytes and not a failure in function of the residual cells. Further, studies in which mice were adrenalectomized prior to subjection to a zinc deficient diet showed that the lymphopenia associated with zinc deficiency was reversed when the GC were removed (DePasquale-Jardieu & Fraker, 1980). In similar studies Wing *et al.* (1988) found that mice starved for 72 hours experienced thymic and splenic atrophy and reduced resistance to infection, while adrenalectomy prior to starvation protected against lymphopenia and infection. Additionally, lymphopenia, along with elevated plasma GC, have been documented among burn patients and, not surprisingly, sepsis is a major cause of death among those who survive the initial injury (Antonacci *et al.*, 1984; Volenec *et al.* 1979; O'Mahony *et al.* 1985; Kagan *et al.* 1989). The lymphopenia and increased incidences of infection associated with chronic stresses may be indicative of alterations in lymphopoiesis. It is known that GC cause atrophy of the immature cells of the thymus (Weissman, 1973), the site of T-cell development. Recently it has been found that GC-induced thymic atrophy is due to activation of an internal cellular program of suicide termed programmed cell death (also known as apoptosis) (Compton & Cidlowski, 1986; Pechatnikov *et al.*, 1986). However, it was unknown whether or not the precursor and immature B-lineage cells which reside in the bone marrow also underwent apoptosis when chronically exposed to levels of GC analagous to those reported as a result of surgery, trauma, malnutrition, etc.

Unpublished data from this laboratory have indicated that zinc deficiency significantly reduces the proportion of B-cell precursors in the murine bone marrow (C. Medina and L. King, unpublished results). These data suggest that, in addition to T-cell lymphopoiesis being impared in the thymus, zinc deficiency (and possibly other chronic stresses) significantly alter B-cell lymphopoiesis which takes place in the bone marrow. Additionally, there have been some reports that injection of large quantities of cortisol or a synthetic GC, dexamethasone, cause a signifcant reduction in B-lineage cells in the murine bone marrow (Ku & Witte, 1986; Sabbele *et al.* 1987). However, the role chronic exposure to physiological concentrations of GC might play in bone marrow B-cell development was unknown.

Therefore, this dissertation project was designed to determine the effects of physiological concentrations of GC on murine B-cell lymphopoiesis. The specific questions addressed in this project were:

1. What effects do GC concentrations, similar to those produced by chronic stress, have on the *in vivo* development of B-cells in murine bone marrow? The few studies which have examined the effects of GC on BM B-cells reported depletion of subpopulations of cells, but at different points along the developmental pathway (Ku & Witte, 1986; Sabbele *et al.*, 1987; Vines *et al.*, 1980). Since these studies used pharmacological doses of GC, the effects of physiological concentrations of GC on B-cell development remained unknown as will be discussed. An *in vivo* delivery system which elevated plasma corticosterone to the levels reported for stressed mice was developed to address this question.

2. Can GC cause apoptosis in the immature B-lineage cells in the murine bone marrow? Since GC have been shown to induce apoptosis in immature T-lymphocytes (Wyllie, 1980; Umansky *et al.*, 1981) it is logical to presume that developing B-lymphocytes in the BM may be affected by chronically elevated GC in a similar manner. However, owing to the heterogeneous nature of the BM, this was a difficult question to answer because of the nature of the current assays commonly used to quantitate apoptosis. A unique method for addressing this question was developed in this laboratory utilyzing DNA binding dyes (Telford *et al.*, 1991) in conjunction with two-color flow cytometry cell cycle analysis as will be demonstrated.

3. What effect do concentrations of GC corresponding to those

found during chronic stress have on bone marrow B-cell function? It is known that GC inhibit the antigenic response of mature B-cells isolated from peripheral lymphoid organs by blocking entry into the cell cycle (Luster *et al.*, 1988; Dennis *et al.*, 1987). However, there was no indication what effect GC might have on the response of BM B-lineage cells to antigen. A short term BM culture system (Medina *et al.*, 1988) was utilized which examined the effects of GC within a physiological range of concentrations on the clonal expansion of BM B-cells in response to the T-cell-independent antigen trinitrophenol-lipopolysaccharide.

4. Do B-lineage cells in murine bone marrow have functional glucocorticoid receptors? A cytosolic GC receptor has been shown to mediate nearly all of the physiological effects of GC. For example, several studies have shown that functional receptors are necessary for GC induced cell death in transformed T-cell lines (Dieken *et al.*, 1990; Harbour *et al.*, 1990). Though the GC receptor has been extensively studied in thymocytes and peripheral lymphocytes, GC receptors had never been directly demonstrated in murine BM B-cells. B-lineage cells were isolated from the murine BM and analyzed for the presence of GC receptors using a whole cell binding assay.

5. Finally, the methods developed herein were used to determine if prednisolone (PD), a widely used very potent immunosuppressor steroid, also altered lymphopoiesis via induction of programmed cell death. Although the majority of

this work was concerned with the effects of natural or endogenously produced GC on the production of new lymphoid cells, the wide pharmacological use of PD made it important to ascertain if it also altered lymphopoiesis in the BM.

The literature relating to the effects of GC on the immune system is quite extensive; therefore, this review will focus only on four main areas. First, an overview of development of cells of B-lineage in the bone marrow will be presented. If GC alters lymphopoiesis it would be important to know if there is a specific site along the developmental pathway of stem, pre-B, or immature B-cells where that occurrs. B-cell development proceeds through a series of distinct stages, some of which are distiguished by cells actively cycling while in other stages the cells are quiescent. GC have been shown to arrest a transformed T-cell line in the G_1 phase of the cell cycle (Harmon *et al.*, 1979). It is possible that if GC have an effect on lymphopoietic processes it will be in the stages of development in which cells are cycling. Therefore, the proliferative capacity of the different stages of B-cell development will be discussed. B-cell lymphopoiesis is at least partially regulated by soluble growth factors produced by cells in the heterogeneous BM microenvironment. Since it is possible that GC could indirectly affect lymphopoiesis by altering the BM microenvironment and the production of essential growth factors, it is important to have an understanding of the environmental elements which regulate B-cell development. Second, aspects of GC biochemistry will be reviewed to include the current knowledge regarding the structure and function of the GC receptor. Third, morphological and biochemical aspects of GC induced apoptosis will be reviewed. Since it is possible that, like thymocytes, immature developing B-lineage cells are induced to undergo apoptosis by GC, it is important to understand the characteristics of programmed cell death. While there is nothing known about GC induced apoptosis in BM B-lineage cells, there has been intense investigation of thymocyte cell death. However, there is evidence from the literature that B-lineage cells possess the necessary biochemical pathways for undergoing apoptosis and these will be discussed. Fourth, the more current literature regarding the effects of GC on B-cell function will be presented which, though dealing primarily with mature peripheral cells, provides evidence that B-lineage cells are sensitive to physiological concentrations of GC. The sparse literature surrounding the effects of GC on BM B-lineage cells also will be discussed.

OVERVIEW OF THE MAJOR CLASSES OF CELLS OF THE BONE MARROW

The immune system is comprised of an array of cells with diverse morphology and function, all of which derive from a small number of self-renewing hematopoietic stem cells in the BM. These pluripotent stem cells give rise to committed stem cells of the erythroid, myeloid, or lymphoid lineages (Figure 1.1). Myeloid progenitors differentiate into monocytes, neutrophils, eosinophils, basophils, or megakaryocytes. Lymphoid-committed progenitors differentiate into T- and B-lineage and natural killer cells. T-cell precursors leave the BM via the peripheral blood and enter the thymic cortex as thymocytes where a small fraction develop into mature medullary T-cells. B-lineage cells in mammals, however, remain in the BM where they develop from precursor B-cells into surface immunoglobulin bearing B-cells that can respond to antigen. Though the BM is heterogeneous with B-lineage cells representing only 20-30% of the total population, their development resides totally in the BM making it easier to follow.

STAGES OF B-CELL DEVELOPMENT: PHENOTYPE, GENE REARRANGEMENT, KINETICS, AND REGULATION

B-cells are the precursors to antibody producing cells of the immune system. Each mature B-cell expresses plasma membrane-bound immunoglobulin (sIg) with a single specificity which acts as an antigen receptor. There are five different isotypes of immunoglobulin molecules in mammals: M (μ), D (δ), G (γ), A (α), and E (ϵ). IgM and IgD appear on fully differentiated but quiescent B-cells which have never been exposed to antigen (known as virgin B-cells). Binding of antigen by sIg in the presence of requisite cytokines results in activation of a signalling pathway involving the hydrolysis of phosphoinositides and activation of protein kinase C (Cambier & Ransom, 1987; Ales-Martinez *et al.*, 1991). The stimulated B-cells differentiate into either antibody-secreting plasma cells or memory B-cells. In adult mammals, B-cells are produced exclusively in the BM and migrate in the mature state to peripheral lymphoid tissues such as the spleen, systemic circulation, lymph nodes, mucosa, Peyer's patches, etc. B-cell development proceeds through a series of distinct stages which can be defined by various phenotypic markers and/or degree of immunoglobulin gene rearrangement and expression, as will be discussed (Kincade *et al.*, 1989; Coffman, 1982).

Phenotypic Markers Associated With Various Stages of B-cell Development in Bone Marrow: Lymphopoiesis

The earliest identifiable cells committed to the B-lineage found in the BM contain the nuclear enzyme, terminal deoxynucleotidyl transferase (TdT) (Gregoire *et al.*, 1977; Park & Osmond, 1987). Less than 4% of nucleated BM cells from adult mice or rats express TdT (Gregoire *et al.*, 1977; Park &

Osmond, 1987; Opstelten *et al.*, 1986) which is thought to play a role in generating immunological diversity by inserting short nucleotide sequences at gene segment junctions created by immunoglobulin heavy chain gene rearrangement (Desiderio *et al.*, 1984; Baltimore, 1974). As a result TdT, while appearing early in B-cell development, is no longer found in cells prior to surface immunoglobulin expression (Figure 1.2).

TdT^+ cells are fairly large with a diameter ranging betweem 6-15 μm (Park & Osmond, 1987, 1989). These early precursors are actively cycling but are not thought to be self-renewing. Studies in which mice were injected with vincristine (to cause arrest of cells in metaphase) made it possible to determine the kinetics of turnover of TdT^+ cells in BM. Park and Osmond (1987, 1989) determined that the rate of entry of TdT^+ cells from mouse BM into mitosis is 5-9%/hour with a cell cycle time of 11-20 hours. They estimated that approximately $7x10^6$ TdT^+ cells per day are produced in the mouse (Park & Osmond, 1989). It has been reported that GC adversely effect this actively cycling population of TdT^+ cells which will be discussed later in this review (Vines *et al.*, 1980; Schrader *et al.*, 1979).

B220 is a 220,000 MW glycoprotein of the leukocyte-common antigen (L-CA) family that has been identified as a unique B-cell molecule which spans the plasma membrane (Coffman, 1981). Molecules in the L-CA family are thought to be a cell surface receptor, though a ligand has not been identified.

Recently, it has been determined that the cytoplasmic domain of these molecules has protein tyrosine phosphatase activity and investigators are actively working to determine the significance of this finding (Thomas, 1989; Ostergaard & Trowbridge, 1991). Antibodies to murine B220 have been generated in several laboratories the most common being 14.8 (Kincade *et al.*, 1981) and RA3.3A1 or RA3.6B2 (Coffman & Weissman, 1981; Coffman & Weissman, 1981). While the 14.8 antibody has cross reactivity with the L-CA form found on $CD8^+$ T-cells (T200), RA3.3A1 and RA3.6B2 have no T-cell cross reactivity (Thomas & Lefrancois, 1988). RA3.3A1 and RA3.6B2 were used extensively in the studies presented in this dissertation for identification and isolation of subpopulations of B-lineage bone marrow cells (Figure 1.2).

B220 appears on all mature B-cells and a population of precursor sIg^- B-cells (Coffman & Weissman, 1981; Kincade *et al.*, 1981) which represent approximately 20-30% of total nucleated BM cells (Landreth *et al.*, 1983; Velardi & Cooper, 1984; Kincade *et al.*, 1981; Park & Osmond, 1987; Coffman & Weissman, 1981) (Figure 1.2). In addition, it was found that B220 is coexpressed with about 50% of TdT^+ cells in mouse BM and all TdT^+ cells in rat BM (Park & Osmond, 1987; Park & Osmond, 1989; Opstelten *et al.*, 1986; Deenen *et al.*, 1990). Mouse TdT^+ $B220^-$ cells represent approximately 0.8% of total nucleated cells, are around 9 μm in diameter, and have a turnover rate of 5.1%/hour (Park & Osmond, 1987; Park &

Osmond, 1989). TdT^+ $B220^+$ (14.8 antibody) are slightly larger than TdT^+B220^- cells with a diameter of around 10 μm and turnover faster at 9%/hour (Park & Osmond, 1989). Cells which are TdT^-B220^+, but do not yet express immunoglobulin (Ig) either in the cytoplasm nor on the cell surface, represent about 4% of total nucleated murine BM, are around 11.5 μm in diameter, and have a turnover rate of 13%/hour (Park & Osmond, 1987; Park & Osmond, 1989). The total production of TdT^+B220^-, TdT^+B220^+ and $TdT^-B220^+Ig^-$ cells in the whole mouse BM is about $3x10^6$, $5x10^6$, and $36x10^6$ cells per day, respectively (Park & Osmond, 1989). Thus, B220 has proven to be a useful phenotypic marker for identifying early stages of B-lineage cell development when coupled with analysis of other key markers. It has been used in the work presented in this dissertation, along with antibodies to sIg, to distinguish precursor from immature and mature B-cells.

Shortly after B220 appears on the surface of B-lineage cells the synthesis of μ immunoglobulin heavy chains is detectable in the cytoplasm (Raff *et al.*, 1976; Levitt & Cooper, 1980). Cytoplasmic μ (cμ) containing cells are all $TdT^-B220^+sIg^-$ and can be further divided into important subsets based on cell diameter and cell cycle status (Landreth *et al.*, 1983; Landreth *et al.*, 1981; Velardi & Cooper, 1984; Opstelten & Osmond, 1983; Osmond & Owen, 1984) (Figure 1.2). Large $c\mu^+$ cells are greater than 9 μm in diameter and have been shown to be actively cycling since they readily incorporate ^{3}H-thymidine and bromodeoxyuridine into their DNA (Landreth *et al.*, 1981; Opstelten & Osmond, 1983; Deenen *et al.*, 1990). Conversely, small cμ^+ cells (less than 9 μm in diameter) are non-cycling but will appear labelled with ^{3}H-thymidine after a lag period suggesting that they are the immediate progeny of the actively cycling population (Figure 1.2) (Opstelten & Osmond, 1983, Landreth *et al.*, 1981; Osmond & Owen, 1984). These cμ^+sIg$^-$ populations are referred to as pre-B cells (Raff *et al.*, 1976) and have been reported to represent 3-12% of total nucleated BM cells (Landreth *et al.*, 1981; Landreth *et al.*, 1983; Raff *et al.*, 1976; Opstelten & Osmond, 1983). Large cμ^+sIg$^-$ cells turnover at a rate of 15%/hour and have an average apparent cell cycle time of 7 hours (Opstelten & Osmond, 1983). There is a 5-fold expansion from the 7×10^6 TdT$^+$ cells produced in the mouse per day to a total production of 35×10^6 large cμ^+sIg$^-$ pre-B cells produced/day (Park & Osmond, 1989; Opstelten & Osmond, 1983). However, subsequently there are only about 15×10^6 small cμ^+sIg$^-$ pre-B cells produced per day (Opstelten & Osmond, 1983). This represents a significant loss in cells during the transition from large to small pre-B cells which is also seen in rat BM (Deenen *et al.*, 1990) and may represent cell death due to non-productive immunoglobulin gene rearrangement (Rolink *et al.*, 1991; Rolink & Melchers, 1991a). Indeed, some investigators have proposed that apoptosis may be the mechanism responsible for deleting these pre-B cells (Rolink *et al.*, 1991). This is significant for the work presented here because it suggests that pre-B cells are capable of undergoing apoptosis and that programmed cell death may play a role in the regulation of lymphopoiesis. Whether or not endogenously produced GC are also able to induce the apoptotic pathway in pre-B cells is also a major question addressed in this dissertation.

Small $c\mu^+sIg^-$ cells will acquire cell surface IgM after a lag of less than 48 hours (Landreth *et al.*, 1981). Experiments in which mice were subjected to sublethal doses of irradiation or ^{3}H-thymidine injections indicate that the time of appearance of small $sIgM^+$ cells occurs about 12-48 hours after the reappearance of small $c\mu^+sIg^-$ precursors (Landreth *et al.*, 1981; Osmond & Nossal, 1974; Yang *et al.*, 1978). Though small $sIgM^+$ cells are not actively cycling, they are rapidly renewed from the pre-B cell pool and represent an immature B-cell type (Miller & Osmond, 1975; Landreth *et al.*, 1981; Osmond & Nossal, 1974). In mice, the total BM production is about 16×10^6 $sIgM^+$ cells/day, which is approximately equal to the output of small $c\mu^+sIgM^-$ (Opstelten & Osmond, 1983). With such a dynamic movement of cells from one stage of development to another, a disruption in the lymphopoietic process would be expected to be detected fairly rapidly. Likewise, specific blocks in this developmental pathway would be denoted by an accumulation of subsets of cells prior to the block. Whether or not GC altered progression, this was examined herein.

Approximately 24-48 hours after expression of sIgM immature B-cells also express sIgD (Aspinall & Owen, 1983; Lala *et al.*, 1979). The proportion of $sIgM^+$ cells in the adult murine BM has been reported to be between 7-15% of which approximately 50% also express $sIgD^+$ (Landreth *et al.*, 1981; Opstelten & Osmond, 1983; Scher *et al.*, 1980; Lala *et al.*, 1979; Kearney *et al.*, 1977). As young $sIgM^+$ $sIgD^-$ cells mature, they increase the number of IgM molecules expressed on the surface (Lala *et al.*, 1979; Osmond & Nossal, 1974) and it may be that when they reach a certain IgM density IgD is also expressed (Lala *et al.*, 1979). This is hard to determine since there is no way of knowing if $sIgD^+$ cells residing in the BM are newly developed or if they are part of the fraction moving through the highly vascularized BM from the blood as it circulates. Though the kinetic experiments utilizing ^{3}H-thymidine as a marker for the rate of renewal of lymphocytes, as already detailed, have indicated that BM B-cells are rapidly renewed and short-lived, a smaller population of slowly renewed, long-lived B-cell populations also exist (Press *et al.*, 1977; Yang *et al.*, 1978; Osmond & Nossal, 1974; Miller & Osmond, 1975; Landreth *et al.*, 1983). Recently, it was reported that in adult mice as much as two-thirds of splenic B-cells are long-lived having a lifetime of weeks to months while a more rapid turnover occurs in adolescent mice (Forster & Rajewsky, 1990). In the BM most

$B220^+$ cells were found to be rapidly renewed but a small population of cells were long-lived and interestingly, these cells had a high density of surface B220 expression determined by intensity of staining with a fluoresceinated antibody (Forster & Rajewsky, 1990). These long-lived cells also were found to be $sIgM^+$ and had a high density of sIgD (Gu *et al.*, 1991). These studies suggested that long-lived B-cells in the BM may be identified by the degree of intensity of staining of fluorochrome-conjugated antibodies to B220 and IgD. Although controversial, this could be an important finding for the studies presented in this dissertation. Should GC impair lymphopoiesis, it may be possible to estimate if rapidly renewed, long-lived, or both are adversely effected.

Relationship of Immunoglobulin Gene Rearrangement to Phenotypic Markers

During B-cell development, the pattern of expression of immunoglobulin coincides with the aquisition of some of the phenotypic markers just discussed (Figure 1.2). Immunoglobulin molecules are made up of four subunits, two heavy chains and two light chains. Heavy chain gene expression occurs prior to light chain expression in the developmental pathway. Completion of heavy chain expression is marked by the appearance of cμ in pre-B cells followed by the appearance of sIgM upon completion of light chain expression (Alt *et al.*, 1981; Levitt & Cooper, 1980).

Antibody diversity (approximately 10^9 different specificities) is accomplished by organization of the genes encoding the immunoglobulin molecule into specific patterns by alternative splicing. Heavy chain genes are arranged in four distinct regions each with a number of different segments: variable (V_H, 250-1000 segments), diversity (D, 12 segments), joining (J_H, 4 segments) and constant (C_H, 5 segments) (Figure 1.3). Light chain genes are arranged similarly but lack a D region. During heavy chain rearrangement one segment of the D region is fused to a J_H region segment in TdT^+ cells prior to development of B220 expression (Coffman, 1982; Sugiyama, 1982; Desiderio *et al.*, 1984; Yancopoulos & Alt, 1986; Rolink & Melchers, 1991a). Rearrangement of a single V_H region segment to DJ_H occurs at about the time of appearance of $B220^+$ cells (Sugiyama *et al.*, 1982; Coffman, 1982) prior to $c\mu$ expression. Large pre-B cells express $c\mu$ if they have successfully rearranged the heavy chain gene. Crippling mutations or V and J segments joined in the wrong translational reading frame could lead to deletion of the cell which is probably removed by BM macrophages (Yancopoulos & Alt, 1986). Recently it has been determined that μ heavy chains are expressed on the pre-B cell surface with surrogate proteins known as V_{preB} and λ_5 prior to light chain rearrangement (Tsubata & Reth, 1990; Karasuyama *et al.*, 1990; Rolink & Melchers, 1991a). Expression of $c\mu$ protein induces the rearrangement of light chains (Iglesias *et al.*, 1991;

Yancopolous & Alt, 1986) which occurs by the joining of V_L and J_L region gene segments during the small pre-B cell ($c\mu^+$) stage of development. Productive rearrangement of either κ or λ light chains leads to expression of sIgM at the immature B-cell stage of development. A summary of gene rearrangement (Figure 1.3) along with its relationship to the phenotypic development of BM B-lineage cells is presented in Figure 1.2.

The Bone Marrow Microenvironment: Role of Stromal Cells

Approximately 20-30% of bone marrow nucleated cells are of the B-lineage while the balance includes other hematopoietic cells as discussed (Figure 1.1) as well as supporting stromal cell types which are fixed within the tissue. Stromal cells are actually multiple cell types which include endothelial cells, reticular cells, preadipocytes, and fibroblasts (Dorshkind, 1990). Transmission and scanning electron microscopy have revealed that lymphopoiesis takes place in venous sinuses within the medullary cavity of the bone and that early B-lineage cells reside in the subosteal areas closely associated with stromal cells (Kincade *et al.*, 1989; Dorshkind, 1990; Weiss, 1981; Jacobsen *et al.*, 1990).

Not surprisingly, cell-cell contact between precursor B-lineage cells and these stromal cells has been shown to be necessary for successful development of B-cells in long term bone marrow cultures (LTBMC). Bone marrow cells seeded in diffusion chambers over established stromal layers would not produce B-lineage cells, while pre-B and B-cells developed when seeded in direct contact with the adherent stromal layer (Kierney & Dorshkind, 1987). These studies suggested that at some stages of development, soluble growth factors are not sufficient for B-cell development and direct contact with stromal cells was required. Recently it has been found that an adhesion molecule found on stromal cell lines may be a ligand for an integrin (VLA-4) on the surface of lymphocytes derived from LTBMCs (Miyake *et al.*, 1991; Miyake *et al.*, 1991a). Antibodies to VLA-4 and a widely distributed cell surface glycoprotein called Pgp-1 found on stromal cells blocked B-cell lymphopoiesis in LTBMCs (Miyake *et al.*, 1990; Miyake *et al.*, 1991). As a result, it now has been postulated that early in development B-lineage precursors require direct contact with stromal cells, but as they progress through the developmental pathway they become independent of direct contact (Era *et al.*, 1991) and may then migrate from the subosteal areas of the bone medullary cavity toward the central sinus (Dorshkind, 1990; Hermans *et al.*, 1989).

The required association of stromal cells with precursor B-lineage cells appears to be critical for development of B-cells. Also critical are stromal cell produced cytokines which promote progression from precursor to mature B-cells. Association with BM macrophages also may be important for B-cell development possibly due to the cytokines they secrete. Macrophages also play a role in phagocytosing improperly produced cells (Weiss, 1981; Gisler *et al.*, 1987). Though not specifically addressed in this dissertation, it is important to remember that GC could indirectly affect B-cell lymphopoiesis by altering elements of the microenvironment such as the ability of stromal cells to produce vital cytokines.

Regulation of B-cell Development by Soluble Factors

Our knowledge of the factors that control B-cell development in the BM is largely inadequate. Some factors have been identified since the advent of long term bone marrow cultures described first by Whitlock *et al.* (1984), however, a large portion of our knowledge is due to the use of cloned or transformed cell lines. While these are useful tools for experimentation, it is not known if they reflect the events of the BM microenvironment. Studying the regulation of BM B-cell development *in vivo* has many inherent problems, however there are some studies in the literature which offer insight into the complexity of control of lymphopoiesis.

A number of years ago, Osmond's laboratory published a series of studies aimed at determining if a negative feedback system was in place that regulated the rate of production of new B-cells. At that time it was thought that soluble factors produced by peripheral B-cells might also regulate B-cell lymphopoiesis. Depletion of mature B-cells in mice by *in vivo* administration of antibodies to IgM had no effect on the absolute number of small lymphocytes produced in the BM suggesting that the end products of B-cell development may not have a regulatory role (Fulop *et al.*, 1983; Opselten & Osmond, 1985). Injection of sheep red blood cells into mice did cause an increase in BM pre-B cell proliferation indicating that exogenous stimulants do effect (most likely indirectly) the kinetics of B-cell lymphopoiesis (Opstelten & Osmond, 1985; Pietrangeli & Osmond, 1987; Park & Osmond, 1991). The number of TdT^{+} and $B220^{+}$ cells actively cycling increased significantly by 3-4 days after immunization (Park & Osmond, 1991). Multiple injections of sheep red blood cells resulted in a sustained elevation of early precursor B-cell production in the BM. The factors involved in this up-regulation have not been identified but may be macrophage-derived since splenectomy or treatment of mice with silica to depress macrophage function abrogated the effects (Park & Osmond, 1991).

There are two classes of soluble factors which regulate B-cell development, those which affect proliferation and those which affect differentiation. Interleukin-4, (IL-4) is a stromal cell derived factor which appears to have diverse effects depending upon the concentrations used, the stage of maturation of the B-lineage cells, and the other growth factors present (King *et al.*, 1988; Peschel *et al.*, 1989; Simons & Zharhary, 1989; Dorshkind, 1990). Conditioned medium containing IL-4 has been reported to be important in promoting the maturation of small pre-B cells to B-cells in long term BM culture systems by inducing expression of immunoglobulin light chains (King *et al.*, 1988). However, when added to cultures containing early B220^{-} B-lineage cells, recombinant IL-4 (rIL-4) caused proliferation but had no effect on maturation to the next stage of development (Peschel *et al.*, 1989). Similarly, rIL-4 was shown to promote proliferation of B220^{+} pre-B cells but did not change the frequency of generation of sIg^{+} cells (Simons & Zharhary, 1989). Taken together, these studies suggest that pure rIL-4 promotes proliferation of B-lineage cells in long term BM cultures. However, when present with other factors such as in conditioned media from stromal cell cultures, IL-4 promotes differentiation of B-lineage cells (King *et al.*, 1988). Further, at very high concentrations, rIL-4 has been shown to inhibit growth of B-lineage cells (Rennick *et al.*, 1987).

IL-1 has been reported to have diverse effects on lymphopoiesis. It has been shown to stimulate the expression of κ-light chains and surface Ig on the chemically transformed pre-B cell line 70Z/3 (Giri *et al.*, 1984). However, it has also been reported that IL-1 promotes myelopoiesis at the expense of lymphopoiesis in cultures which have been switched from myeloid to lymphoid conditions (Dorshkind, 1988). Injection of IL-1 *in vivo* has been reported to stimulate myelopoiesis and suppress lymphopoiesis (Dorshkind, 1990). Interestingly, there is evidence that IL-1 and GC form a feedback loop in which IL-1 stimulates GC release from the adrenal glands while GC inhibits IL-1 production by macrophages and other cell types (Dinarello, 1988; Besedovsky *et al.*, 1986). Recently it was reported that physiological concentrations of GC and granulocyte-colony stimulating factor synergistically upregulated IL-1 receptors in a heterogeneous population of BM cells (Shieh *et al.*, 1991). These studies also imply that GC may play an important role in the regulation of hematopoiesis.

Interleukin-7 (IL-7) is a B-lineage cell proliferative factor which was cloned from stromal cell DNA (Namen *et al.*, 1988; Namen *et al.*, 1988a; Goodwin *et al.*, 1989; Henney, 1989). IL-7 promotes the proliferation of large pre-B cells bearing B220 and BP-1 but not their differentiation into immature B-cells (Lee *et al.*, 1989; Williams *et al.*, 1990). Additionally, IL-7 appears to promote survival of purified B220$^+$sIgM$^-$ cells in culture for several weeks, otherwise these cells usually die fairly rapidly without a supportive stromal layer of cells (Lee *et al.*, 1989). Experiments in which IL-7 was injected into either normal or cyclophosphamide treated mice caused an increase in cellularity of the spleen, lymph nodes, thymus, and bone marrow (Morrissey *et al.*, 1991; Morrissey *et al.*, 1991a). In the BM there was a significant expansion in the proportion of pre-B cells which were B220$^+$sIgM$^-$ and cμ^+sIgM$^-$ in the IL-7 treated mice.

Not all stromal cell lines which have been cloned are capable of producing IL-7 (Sudo *et al.*, 1989). These non-IL-7-producing stromal cell lines apparently were not able to support B-cell lymphopoiesis unless exogenous IL-7 was added to the cultures (Sudo *et al.*, 1989; Era *et al.*, 1991; Rolink *et al.*, 1991). However, Sudo *et al.* (1989) and Era *et al.* (1991) proposed a model of B-cell development in which a very early precursor is dependent on stromal cells but not on IL-7. This would be followed by a stage in which the cells are both stromal cell and IL-7-dependent and then by a stromal cell independent, IL-7-dependent stage. Further, Era *et al.* (1991) suggested that the signals that drive these cells to change their growth requirements are determined by the stage of gene rearrangement and expression of the immunoglobulin molecule. Regardless, it is apparent that the effectiveness of IL-7 in promoting B-cell lymphopoiesis is limited to precursor B-lineage cells.

Interestingly, cloning of the IL-7 receptor from a pre-B cell line (70Z/3) has revealed that the promoter region of the gene contains a functional interferon regulatory unit and potential multiple GC response elements (Pleiman *et al.*, 1991). The significance of these response elements is unknown at present but presents interesting possibilities regarding the control of IL-7-induced precursor B-cell proliferation. Since GC are known to alter transcription it is possible that GC have a significant role in regulation of the expression of the IL-7 receptor. Again, while not specifically addressed in this dissertation, this is additional evidence that the *in vivo* effects of GC on B-cell development in BM may be extremely complex.

GLUCOCORTICOIDS AND THE STRESS AXIS

The release of glucocorticoids from the adrenal glands is controlled centrally through the hypothalamic-pituitary-adrenal cortex stress axis (Figure 1.4). Signals originating from a number of central nervous system sources are integrated in the hypothalamus which in turn signals the pituitary gland by releasing corticotropin-releasing factor (CRF) into the portal vein. CRF causes specialized cells in the anterior pituitary to secrete adrenocorticotropic hormone(ACTH) into the systemic circulation. ACTH works through a plasma membrane receptor in the zona fasciculata of the adrenal cortex to stimulate secretion of cortisol and corticosterone. The major effect of ACTH is to stimulate the conversion of cholesterol to pregnenolone by a desmolase complex (including cytochrome P_{450scc}) which is the rate-limiting reaction in the GC synthesis pathway (Bondy & Rosenberg, 1980; Smith *et al.*, 1983) (Figure 1.5).

Cortisol (also known as hydrocortisone) is the most prevalent GC in man making up about 80% of the circulating levels while corticosterone is a minor component. However, in mice and rats, corticosterone is the only circulating GC since they lack 17α-hydroxylase (Spackman & Riley, 1978; Simpson & Waterman, 1988). Plasma GC concentrations undergo diurnal variation with peak cortisol levels of 15-20 μg/dl (about 0.5 μM) plasma around 8 a.m. in non-stressed humans (Berne & Levy, 1983; Smith *et al.*, 1983). In rodents, which are nocturnal, peak corticosterone concentrations (around 20 μg/dl plasma) are reached between 7-10 p.m. (Spackman & Riley, 1978). Therefore, in the studies herein all samples were collected between 8-9 a.m., the lowest point in the diurnal murine rhythm. Chronic stress such as zinc deficiency in mice has been reported to create levels of corticosterone near 100 μg/dl (3 μM) (DePasquale-Jardieu & Fraker, 1980).

Both cortisol (HC) and corticosterone (CS) bind to the plasma protein corticosteroid binding globulin (also known as transcortin) and more weakly to albumin upon release into the systemic circulation. Approximately 90% of the circulating GC is bound to plasma proteins. It is the free steroids that have been thought to be biologically active (Hammond, 1990; Faict *et al.*, 1985). However, this appears to be a controversial point since other investigators have reported that HC and transcortin-bound HC had similarly decreased the proportion of circulating lymphocytes in rats, decreased human peripheral blood lymphocyte response to phytohemagglutinin, and increased rat liver tyrosine aminotransferase activity (Rosner & Hochberg, 1972; Faict *et al.*, 1983). Some investigators have suggested that transcortin actually delivers GC to target tissues (Rosner, 1990; Rosner *et al.*, 1986; Hsu *et al.*, 1986). There does not appear to be a clear function assigned to transcortin or its role regarding the activity of plasma GC. It does appear that pharmacological amounts of GC and hypercorticism depress transcortin concentrations (Rosner, 1990; Vermeulen, 1986) and so chronic stress may have the same effect. It has been reported that during chronic stress the plasma binding sites for GC are saturated and the free levels increase (Hamanaka *et al.*, 1970; Brien, 1981). The method used to measure plasma corticosterone in this dissertation reportedly analyzes free or unbound GC (Mattingly, 1962).

Synthetic GC developed for pharmacological use have been reported to have more potent biological activities than the natural GC. Some of the most common include dexamethasone (DX), prednisolone, prednisone, and triamcinolone acetonide (Figure 1.6). Of these, prednisolone and prednisone most resemble the natural GC and, unlike dexamethasone or tramcinolone acetonide, will bind to CBG (Brien, 1981). A commonly used synthetic GC receptor antagonist is also shown in Figure 1.6. Though the focus of this dissertation was to determine the effects of physiologically relevant natural GC on B-cell development, it was nevertheless desirable to use low concentrations (1 μM or lower) of DX in the initial *in vitro* studies to determine if an effect could be seen. DX has the advantage of not binding to serum proteins in culture and having an activity which has been reported to be 30 times greater than cortisol (Ballard *et al.*, 1975; Berne & Levy, 1983). The effects of prednisolone on B-cell development and function also was examined as a collaborative effort with Bryan Voetberg since prednisolone is perhaps the most widely used GC pharmacologically as will be discussed in chapter 6.

Steroid hormones exert their effects through cytosolic receptors which, upon translocation to the nucleus, have been shown to alter gene transcription (Beato, 1989; Beato *et al.*, 89; Burnstein & Cidlowski, 1989; Bellingham & Cidlowski, 1988). Structurally, the glucocorticoid receptor (GcR) is a 94 kD phosphoprotein which in the rat is composed of 795 amino acids (Miesfeld *et al.*, 1986). The GcR is composed of a ligand-binding domain on the carboxyl terminal side, a centrally located DNA-binding region, and a modulatory domain at the amino terminal half of the molecule (Figure 1.7). The DNA binding region of the GcR has two zinc-binding regions which form "zinc fingers" (Figure 1.7). Each zinc atom is tetrahedrally coordinated by four cysteine residues to form a protein loop (or finger) which interacts with DNA to regulate transcription (Hard *et al.*, 1990; Vallee *et al.*, 1991). The presence of zinc in the putative zinc finger areas of the GcR protein has been shown to alter secondary structure and is necessary for binding to DNA (Archer *et al.*, 1990; Hutchison *et al.*, 1991). Studies using point mutations in the amino-terminal zinc finger suggest that it is responsible for the specificity of binding of the GcR to DNA sequences called glucocorticoid response elements (GRE) (Archer *et al.*, 1990; Danielson *et al.*, 1989). The GRE is a 15-mer consensus sequence located in the promoter region of GC sensitive genes (Figure 1.7) (Beato, 1989). The activated GcR binds as a dimer to DNA with the amino-terminal domains involved in determining binding specificity (Eriksson & Wrange, 1990).

Translocation of the GcR to the nucleus is dependent on hormone binding to the receptor and may involve nuclear localization signals found in the hormone binding and DNA binding domains (Picard & Yamamoto, 1987). Before translocation to the nucleus, the GcR is complexed to a 90 kD heat shock protein (hsp90) which is lost upon activation by ligand binding (Mendel *et al.*, 1986; Dalman *et al.*, 1989; Housley *et al.*, 1990). Though the function of hsp90 has not been determined, recent evidence from a cell-free system suggested it has at least one binding site in the steroid binding domain of the GcR and may protect the GcR from proteolytic cleavage (Housley *et al.*, 1990). Picard *et al.* (1990) reported that in cells which had low levels of hsp90, free receptors failed to enhance transcription upon hormone addition suggesting that hsp90 has an active role in GcR activation.

Several studies have shown that the presence of functional GcR is necessary for GC-induced apoptosis in transformed T-cell lines (Harbour *et al.*, 1990; Dieken *et al.*, 1990). The GcR DNA-binding region has been shown to be absolutely necessary for GC-induced death (Harbour *et al.*, 1990). However, cells which had been transfected with receptor constructs in which the GcR-DNA binding domain was fused to the ligand-binding domain of the estrogen receptor were induced to cell death when exposed to estradiol, indicating that any ligand that would activate the receptor would induce death if the GcR DNA-binding domain was intact (Harbour *et al.*, 1990). The amino-terminal immunomodulatory domain is also critical for GC-induced cell death since defective transcription was observed in cells transfected with GcR constructs lacking the amino-terminal domain (Dieken *et al.*, 1990). Since the presence of functional GcR are obviously critical for the mediation of GC effects in lymphocytes it was important to determine if receptors could be detected in bone marrow B-lineage cells, a question addressed in chapter 5 of this dissertation.

GLUCOCORTICOID EFFECTS ON B-LYMPHOCYTES

Since GC were found to be powerful pharmacological agents widely used against inflammation, autoimmune disease, arthritis, lymphocytic cancers, etc., a number of investigators in the 1970's published reports concerning the effects of GC on immune function. Many of these studies used either acute doses, synthetic GCs, or both while ignoring the potential effects the endogenous GC may have on immune function. Thus whether or not the findings in these studies represent the effect of the physiologically relevant GC released during chronic stress is not certain. Further, many of these studies presumed that T-cells, particularly thymocytes, were much more sensitive to GC than B-lineage cells. Very few studies addressed the possible effects GC might have on developing BM B-cells though this would have been a logical extension given the effects of GC that have been reported on developing T-cells. Therefore, one of the major objectives of this dissertation was to determine the effects of a physiological range of GC concentrations on B-cell lymphopoiesis using both *in vitro* and *in vivo* systems of analysis. Owing to the enormity of the literature on the effects of GC on the immune system, this review will be limited to a discussion of the effects of GC on B-lymphocytes.

Glucocorticoid Effects on Bone Marrow B-cells

The development of long term bone marrow cultures (LTBMC) provided insight into the effects of GC on B-cell development. Culture systems which promoted the production of myeloid lineage cells required the presence of 0.1 μM cortisol (Dexter conditions) while optimal conditions for lymphocyte production required the ommission of HC from culture (Whitlock-Witte conditions) (Whitlock *et al.*, 1884; Whitlock *et al.*, 1987; Hayashi *et al.*, 1984). Glucocorticoids inhibited the production of TdT^{+} cells in LTBMCs, but apparently more primitive B-cell progenitors were GC resistant since switching cultures from Dexter to Whitlock-Witte conditions caused a shift from myelopoiesis to lymphopoiesis (Dorshkind, 1986; Vines *et al.*, 1980; Hayashi *et al.*, 1984; Ku & Witte, 1986). Additionally, GC may influence the BM microenvironment since it has been found that a cloned preadipocyte stromal cell line differentiated to adipocytes upon exposure to HC (Gimble *et al.*, 1990). This cell line was able to support B-lineage cell proliferation in both the preadipocyte and adipocyte form. In spite of this circumstantial evidence that GC may play a role in the regulation of B-cell lymphopoiesis, the effects that physiological concentrations of GC might have on normal B-cell development until now have remained unexplored.

Administration of pharmacological doses of GC *in vivo* does not appear to drastically effect BM cellularity (Fauci, 1975; Ku & Witte, 1986; Sabbele *et al.*, 1987); however, there was some indication that pre-B cells may be adversely effected. Fauci (1975) reported that the number of "null" lymphocytes was decreased in guinea pig BM after a single injection of 10 mg HC. These "null" lymphocytes were probably precursors which did not bear sIg. Ku and Witte (1986) determined that the proportion of $c\mu^+$ cells was significantly reduced in the murine BM after a single injection of 15 mg HC, while Sabbele *et al.* (1987) reported that seven daily injections of 1 mg DX caused a 50% drop in murine BM B-cells. Vines *et al.* (1980) reported that a single injection of 1 mg DX into rats resulted in a significant reduction in large (greater than 8.5 μm) BM lymphocytes and a 10-fold decrease in BM TdT activity. These studies provide significant evidence that GC alter B-cell development. However, all used either acute or pharmacological doses of GC. There is no indication as to the effects of physiological concentrations of endogenous GC on developing B-cells in the BM.

Shifts in Trafficking of Lymphocytes Created by Glucocorticoids

Both chronic and acute doses of GC have been shown to cause a transient decrease in circulating lymphocytes and monocytes in humans, guinea pigs, rats, and mice (Fauci & Dale, 1975; Fauci & Dale, 1975a; Fauci, 1975; Fauci, 1976; Balow *et al.*, 1975, Stevenson & Taylor, 1988, Haynes *et al.*, 1979, Cox & Ford, 1982). Maximal lymphopenia occurs about 4 hours after single or alternate-day injections of PD or HC, but peripheral blood lymphocytes (PBL) return to normal by 24 hours (Fauci & Dale, 1975; Fauci, 1976; Yu *et al.*, 1974). It generally has been thought that these PBL are redistributed to the BM immediately after administration of GC (Fauci, 1975; Cohen, 1972); however, the evidence for this is not particularly compelling. Fauci (1975) reported an increase in the proportion and absolute numbers of T and B-cells in guinea pig BM after a dose of HC which caused plasma HC levels to increase 12-fold to nearly 300 μg/dl. However, BM cellularity was unchanged and there was no attempt to determine if any changes had taken place in the precursor populations. Also, plasma HC levels were extremely high and had no relationship to physiological stress levels which this dissertation addressed. ^{51}Cr-labelled lymphocytes injected intravenously just prior to GC treatment have been used in an effort to identify cells recirculating to the BM (Fauci & Dale, 1975; Fauci, 1975). In one experiment the radioactivity recovered in the BM of cortisone treated guinea pigs was slightly higher (11.9%) than in saline injected animals (9.4%) at 4 hours. Twenty four and 48 hours later the radioactivity recovered in the cortisone treated animals was about 40% greater than in controls, but the difference was due to a drop in the saline-treated animals and not increased accumulation of recirculated cells in the BM. If anything, these data suggested that cells were not leaving the BM, rather than being redistributed there. A more convincing approach to identifying redistributed cells in mice was to transfer BM from mice receiving 5 daily injections of 5 mg HC to irradiated recipients and measure their response to antigenic challenge (Cohen, 1972). Cohen (1972) concluded that peripheral blood T-cells redistributed to the BM since mice which received BM from HC-treated mice responded to challenge by a T-cell-dependent antigen while BM cells transferred from control mice did not respond. Since mature T-cells are rare in the BM due to migration of precursors to the thymus for completion of development, this study provided evidence that there may be some redistribution of peripheral T-cells to the BM. However, the dose of GC used was quite large and there was no evidence presented that B-cells were redistributed. Thus the major oversight in the studies which suggest peripheral blood lymphocytes redistribute to the BM upon administration of pharmacological doses of GC was the failure to examine BM B-lineage cells in various stages of development.

B-cell Function Subsequent to Exposure to GC

Though chronic stress causes lymphopenia, residual cells have been reported to function normally (DePasquale-Jardieu & Fraker, 1980). However, *in vitro* studies have suggested that GC suppress the antigenic response of both human and murine peripheral, mature B-cells (Luster *et al.*, 1988; Bowen & Fauci, 1984; Cupps *et al.*, 1985). There has been very little information regarding the effects of GC on the function of BM B-cells. A brief review of the effects of GC on B-cell function is provided here since this dissertation addresses the effects of GC on BM B-cell responses to an antigen both *in vitro* and *in vivo*.

The spontaneous production of immunoglobulin by unactivated B-cells has been reported to be either increased, decreased, or unchanged depending on the experimental conditions (Cupps, 1989; Grayson *et al.*, 1981; Orson & Auzenne, 1988; Saxon *et al.*, 1978; Cupps *et al.*, 1984; Wira *et al.*, 1990, Sierakowski & Goodwin, 1988). Addition of GC to cultures of human peripheral blood mononuclear cells (PBMNC) has resulted in the increased production of Ig-secreting cells (Grayson *et al.*, 1981; Orson & Auzenne, 1988). This increase was similar to cultures stimulated with PWM and required the presence of monocytes. However, while proliferation also was increased in cells stimulated with PWM, treated cells with 10μM HC (a pharmacological concentration) did not proliferate (Grayson *et al.*, 1981). These data suggested that the spontaneous release of Ig was a nonspecific response and not due to cellular activation. Administration of GC *in vivo* has been reported to have varying effects on Ig synthesis. Production of IgG, IgM, and IgA was diminished in human peripheral blood cells 4 hours after a single injection of methylprednisolone (Saxon *et al.*, 1978), while in another study spontaneous Ig-producing cells were increased (Cupps *et al.*, 1984). While the doses of prednisolone were similar in these studies, the methods for detecting Ig were vastly different; one measured Ig-producing cells while the other attempted to measure soluble Ig after a brief culture period. The differences in the literature regarding the effects of GC on immunoglobulin production may be due to the differences in experimental protocols, particularly the GC used and the dose administered.

The literature is for the most part in agreement regarding the suppressive effects of GC on mature peripheral B-cell responses to mitogenic and antigenic stimulation *in vitro*. Several investigators reported that a proliferative response by Ig-producing cells to the B-cell mitogen lipopolysaccharide was suppressed when GC were added to murine splenic cultures (Roess *et al.*, 1982; Roess *et al.*, 1983; Benner *et al.*, 1979; Sabbele *et al.*, 1987; Luster *et al.*, 1988). Interestingly, when GC were added 24 hours after the mitogen in these cultures, there was no suppressive effect. This suggested that GC affected early events in the stimulation of the cells (Roess *et al.*, 83). Similarly, several studies have shown that early events associated with antigenic activation of mature murine splenic as well as human tonsillar and peripheral blood B-cells are sensitive to GC *in vitro* (Luster *et al.*, 1988; Bowen & Fauci, 1984; Cupps *et al.*, 1985; Dennis *et al.*, 1987). Bowen and Fauci (1984) used cell size and cell cycle-dependent activation markers on human tonsillar B-cells to determine that HC inhibited the progression of anti-μ-treated cells from G_0 to G_{1a} or G_{1b} in the cell cycle. These cells did not proliferate in response to anti-μ nor *Staphylococcus aureus* Cowan strain I (SAC) in the presense of moderate concentrations of HC. However, preactivated cells were not effected by HC (Bowen & Fauci, 1984). Similar results were reported for human peripheral blood B-cells exposed to HC along with SAC or anit-μ (Cupps *et al.*, 1985). Addition of HC 48 hours after stimulation failed to inhibit the proliferative effects of SAC (Cupps *et al.*, 1985). The response of mouse splenic B-cells to anti-Ig or a T-cell-independent antigen (TNP-LPS) was significantly suppressed by DX (Dennis *et al.*, 1987; Luster *et al.*, 1988). Using acridine orange fluorescence to determine cell cycle status, Luster *et al.* (1988) found that DX caused an accumulation of anti-Ig-treated cells in G_0, and decreased expression of maturation-associated antigens including Ia expression (Dennis & Mond, 1986; McMillan *et al.*, 1988; Luster *et al.*, 1988). Addition of DX later than 48 hours after anti-Ig stimulation had no effect on IgM secretion in splenic B-cell cultures while addition of DX up to 48 hours suppressed the response (Luster *et al.*, 1988); which once again suggested that GC inhibited early events in the stimulation process. It appears that DX inhibits phosphoinositide hydrolysis which is an early event in B-cell activation (Dennis *et al.*, 1987; Luster *et al.*, 1989), however, DX has no effect on B-cells stimulated with phorbol myristic acid and A23187 (a calcium ionophore) (Dennis *et al.*, 1987). Interestingly, phorbol esters have been shown to inhibit GC induced apoptosis in thymocytes (McConkey *et al.*, 1989b) as will be discussed later in this review. Taken together, these studies indicate that GC directly affect B-cells by interfering with the signalling process somewhere between Ig receptor cross-linking and protein kinase C activation. These represent very early events in B-cell activation (DeFranco *et al.*, 1987). While it has been fairly well established that GC inhibit early events in activation of peripheral B-cells, there was little information as to whether precursor and immature BM B-cells exhibited similar sensitivity, a question this dissertation addresses.

Glucocorticoids present within the first few hours have been shown to have a suppressive effect on B-cells presented with an antigenic challenge. Injection of HC or CS several days before primary immunization with SRBC has been shown to suppress the production of Ig-secreting cells (or plaque forming cells, PFC) in the murine spleen (Dracott & Smith, 1979; Stevenson & Taylor, 1988). Additionally, daily injections of DX starting one day before or five days after a booster injection of SRBC suppressed the IgM, IgG, and IgA PFC response in murine splenocytes (Benner *et al.*, 1978). These differences were even more striking when expressed as PFC/spleen since splenic atrophy had taken place in the GC-treated mice (Benner *et al.*, 1978). As noted for lipopolysacharide-stimulated mice (Benner *et al.*, 1979, Sabbele *et al.*, 1987), BM appeared to be resistant to the effects of GC since SRBC-stimulated IgM and IgG PFC production was not different than in controls (Benner *et al.*, 1978; Dracott & Smith, 1979). These studies suggested that acute GC administration *in vivo* significantly reduced the immune response of peripheral B-cells but had no effect on the response of BM B-lineage cells. Most of these studies used pharmacological doses of GC, however one study reported that injection of 2.5 mg HC into mice resulted in a transient increase in plasma HC to near 50 μg/dl (Dracott & Smith, 1979), a concentration found in stressed animals (DePasquale-Jardieu & Fraker, 1980). While the BM in these

HC-treated mice responded to antigenic challenge normally (Dracott & Smithe, 1979a), the GC levels were elevated for only 2 days. This dissertation addressed the effect of chronic elevation of GC on BM B-cell function.

GLUCOCORTICOID INDUCED APOPTOSIS

It has been known for some time that GC cause thymic atrophy in mice (Claman *et al.*, 1971; Weissman, 1973). This atrophy was found to be more pronounced in the thymic cortex where the more immature thymocytes reside (Weissman, 1973). More recently, it has been determined that GC cause a selective loss of a subpopulation of immature thymocytes bearing the surface molecules $CD8^{+}/CD4^{+}$ (Screpanti *et al.*, 1989). There has been some question as to the relevance of these findings to humans since it is thought that humans are relatively "glucocorticoid resistant" as are guinea pigs (Claman *et al.*, 1971). Early studies found that mouse thymocytes underwent "cytolysis" upon *in vitro* exposure to GC while human and guinea pig thymocytes were unaffected (Claman *et al.*, 1971). However, it appeared as though human prothymocytes (early precusors which migrate from the BM) experience cytolysis upon exposure to GC as do activated periperal blood T-cells in the mixed lymphocyte reaction. This suggested that subpopulations of human lymphocytes were susceptible to GC-mediated death (Galili, 1983). More recently it has been determined that GC do not directly kill thymocytes but induce the activation of an internal cascade which induces thymocytes to undergo suicide (Wyllie, 1980). This form of cellular suicide has been termed programmed cell death or apoptosis and is a widespread phenomenon with many inducers now identified, chief among them being GC (Wyllie *et al.*, 1980a). These findings provide provocative possibilities regarding the effects of GC on developing B-cells and regulation of lymphopoiesis in normal and stress conditions. If physiological levels of GC altered lymphopoiesis by reducing the proportion of developing B-cells, induction of apoptosis would be a prime candidate for the mechanism of depletion. This issue was addressed in the experiments of the dissertation, therefore, it is important to have an understanding of the morphological and biochemical characteristics of apoptosis.

Morphological Characteristics of Programmed Cell Death

The morphological and ultrastructural changes associated with apoptosis are quite distinct from necrotic death. Necrosis, which is caused by environmental insults such as hypoxia, extreme hyperthermia, and pH changes is characterized by cellular swelling which eventually leads to rupture of nuclear, organelle, and cytoplasmic membranes (Wyllie *et al.*, 1980, Kerr *et al.*, 1987). DNA fragmentation occurs late in the necrotic process and may be caused by lysosomal enzymes released as a result of membrane rupture (Wyllie *et al.*, 1980).

Rather than swelling, apoptotic cells tend to condense their cytoplasm and chromatin leading to a increased buoyant density (Telford *et al.*, 1991; Wyllie & Morris, 1982; Wyllie *et al.*, 1980; Kerr *et al.*, 1987). The nuclear membrane becomes convoluted, indented, and then fragmented. DNA is fragmented into multiples of 180-200 base pairs relatively early in the apoptotic process since fragmentation is detected prior to loss of viability (Wyllie, 1980, Umansky *et al.*, 1981; Telford *et al.*, 1991). The increased density and DNA fragmentation of apoptotic cells were important characteristics which were utilized in this dissertation so that flow cytometry could be used to detect apoptosis.

Plasma membrane blebbing takes place when cytoplasmic protuberances emanate from the cell membrane to form "apoptotic bodies" (Wyllie, 1980a; Wyllie *et al.*, 1980). However, membrane blebbing may not be particularly prominent in cells with a high nucleus-to-cytoplasm ratio such as thymocytes (or B-lymphocytes) (Kerr *et al.*, 1987). Membrane-bound apoptotic bodies which contain cytoplasm and possibly some nuclear material are rapidly phagocytosed by adjacent cells (Duvall *et al.*, 1985; Savill *et al.*, 1990). This is particularly important in the BM where phagocytic cells are abundant and the opportunity to study apoptosis could be impaired by rapid removal of apoptotic cells.

Biochemical and Molecular Events

The thymus has become an important tissue in the study of the biochemical mechanism of apoptosis since it is 95% lymphocytes, of which a significant proportion can be induced to enter apoptosis at one time. Since virtually nothing is known regarding GC-induced apoptosis in B-cells, this discussion will be centered around thymocytes. A number of inducers can stimulate the death pathway in thymocytes including GC, ionizing irradiation, and antibodies to the T cell receptor (Wyllie, 1980; Sellins & Cohen, 1987; Smith *et al.*, 1989). However, owing to the exquisite sensitivity of thymocytes to GC, much of the early work regarding the biochemistry of apoptosis was done using various forms of prednisolone in thymocyte culture systems. In the few studies in which thymic apoptosis was induced *in vivo*, single pharmacological doses of DX (1 μg to 1 mg in rats) or HC (4-10 mg in mice and rats) were used (Compton & Cidlowski, 1986; Pechatnikov *et al.*, 1986; Swat *et al.*, 1991). However, little was known about whether chronic elevation of plasma GC at concentrations resembling those seen during stress represented a significant inducer of apoptosis in thymocytes or other immature lymphocytes. That important question also was addressed in this dissertation.

The earliest event in the process of GC-induced apoptosis is the binding of the ligand to its cytoplasmic receptor. Antagonism of the GC receptor (GcR) resulted in inhibition of

DNA fragmentation in thymocytes exposed to GC (Compton & Cidlowski, 1986; Compton *et al.*, 1988, Telford *et al.*, 1991). Further, studies utilizing various GcR gene constructs transfected into transformed cell lines revealed that functional receptors were necessary for GC induction of apoptosis (Harbour *et al.*, 1990, Dieken *et al.*, 1990). Binding of GC to functional receptors resulted in alteration of gene transcription (Burnstein & Cidlowski, 1989; LaPointe & Baxter, 1989). However, the gene(s) associated with induction of apoptosis have not been identified.

Wyllie first reported that GC-induced apoptotic thymocytes undergo an internucleosomal degradation of DNA (Wyllie, 1980). Nucleosomes are composed of 146 base pairs of DNA wrapped around octamer complexes of core proteins called histones, followed by approximately 50 base pair linker regions which connect adjacent nucleosomes (Figure 1.8). The linker regions between nucleosomes are particularly susceptible to cleavage by nucleases which produce fragments of 200 base pair multiples which can be visualized on agarose gels subsequent to extraction of the cellular DNA (Wyllie, 1980, Umansky *et al.*, 1981). Recently, it also has been shown that intact cells undergoing DNA fragmentation can be quantitated using fluorescent DNA probes and flow cytometry (Pechatnikov *et al.*, 1986; Compton *et al.*, 1988; Telford *et al.*, 1991). This method has a decided advantage over agarose gels for assessing apoptosis in heterogeneous populations of cells such as BM as will be discussed.

Cohen and Duke (1984) determined that the endogeneous endonuclease is dependent on Ca^{2+} and Mg^{2+}. They found that in the absense of GC, Ca^{2+}, and Mg^{2+} induced nuclei isolated from thymus, spleen, and lymph nodes (but not BM) to undergo DNA fragmentation (Cohen & Duke, 1984). Interestingly, high concentrations of zinc were shown to inhibit GC-induced DNA fragmentation, though the significance of this finding currently is unknown (Cohen & Duke, 1984; Nieto & Lopez-Rivas, 1989; Telford *et al.*, 1991). Inhibitors of transcription and protein synthesis have been shown to abrogate GC-induced DNA fragmentation and cytosolic Ca^{2+} changes in thymocytes (McConkey *et al.*, 1990b; McConkey *et al.*, 1989a; Cohen & Duke, 1984). However, since some of these inhibitors also have been shown to induce DNA fragmentation in a mouse lymphoma line (Vedeckis & Bradshaw, 1983) it appears that they merely delay the GC-induced process. These observations have led to a search for the "death proteins" which appear to be induced by GC (Colbert & Young, 1986, Compton & Cidlowski, 1987; Owens, *et al*. 1991). Compton and Cidlowski (1987) identified two protein families which they claimed were GC induced nucleases; however, Alnemri and Litwack (1989) later refuted these findings and suggested that the endogenous endonuclease is not newly synthesized but activated by GC. Van den Bogert *et al.* (1989) identified six nuclear proteins expressed within a short time after exposure to GC but determined that they were histones. By comparing cDNA libraries generated from both untreated and GC-treated thymocytes, Owens *et al.* (1991) identified several mRNAs whose protein products may be important in apoptosis. One of these mRNAs, RP-8, may have DNA-regulatory activity since sequence analysis suggested the presence of a zinc-binding domain (Owen *et al.*, 1991).

An early event in induction of the apoptotic pathway is a large influx of Ca^{2+} into the cytosol (McConkey *et al.*, 1989; McConkey *et al.*, 1989a; Kizaki *et al.*, 1989). McConkey *et al.* (1989a) determined that within 90 minutes of exposure to 10 μM methyl-PD the cytoplasmic Ca^{2+} concentration of rat thymocytes had increased eight fold. Buffering of internal Ca^{2+} by preloading cells with quin-2 (a calcium chelator) resulted in abbrogation of GC-induced DNA fragmentation while the calcium ionophore, A23187, overwhelmed the buffering capacity and restored endonuclease activity (McConkey *et al.*, 1989a). Ca^{2+} was found to enter the cytoplasm from external fluid since use of calcium-depleted media or external chelators resulted in failure of GC (or A23187) to induce increased cytoplasmic Ca^{2+} levels (McConkey *et al.*, 1989a; Kizaki *et al.*, 1989). It appears this GC-stimulated Ca^{2+} influx is not dependent on pre-existing channels since Ca^{2+} channel blockers did not inhibit the increase in cytoplasmic Ca^{2+} concentration (McConkey *et al.*, 1989a). The intermediate events between GC binding and Ca^{2+} influx are not known and the exact role Ca^{2+} plays in apoptosis has not been elucidated. However, it has been shown that the endonuclease induced in thymocytes is Ca^{2+}- and Mg^{2+}-dependent (Cohen & Duke, 1984; McConkey *et al.*, 1989b; McConkey *et al.*, 1989; Nieto Lopez-Rivas, 1989). Whether Ca^{2+} has other functions in the apoptotic pathway is unknown.

Activation of protein kinase C (PKC) by phorbol esters also has been shown to inhibit DNA fragmentation induced by calcium ionophores (Kizaki *et al.*, 1989) and methyl-PD (McConkey *et al.*, 1989b). Some investigators have suggested that PKC activation acts as a second signal that leads to cell proliferation rather than cell death (McConkey *et al.*, 1990a; McConkey *et al.*, 1989c). IL-1 and IL-2 have been shown to protect thymocytes from GC induced DNA fragmentation possibly by contributing a second signal or triggering another signalling pathway which may activate PKC (McConkey *et al.*, 1989c; Nieto & Lopez-Rivas, 1989; Fernandez-Ruiz *et al.*, 1989). However, other studies have demonstrated that PKC activation may be a positive signal for apoptosis (Ojeda *et al.*, 1990; Smith *et al.*, 1989). The conflicting results may have to do with differences in phorbol ester concentrations used in these studies.

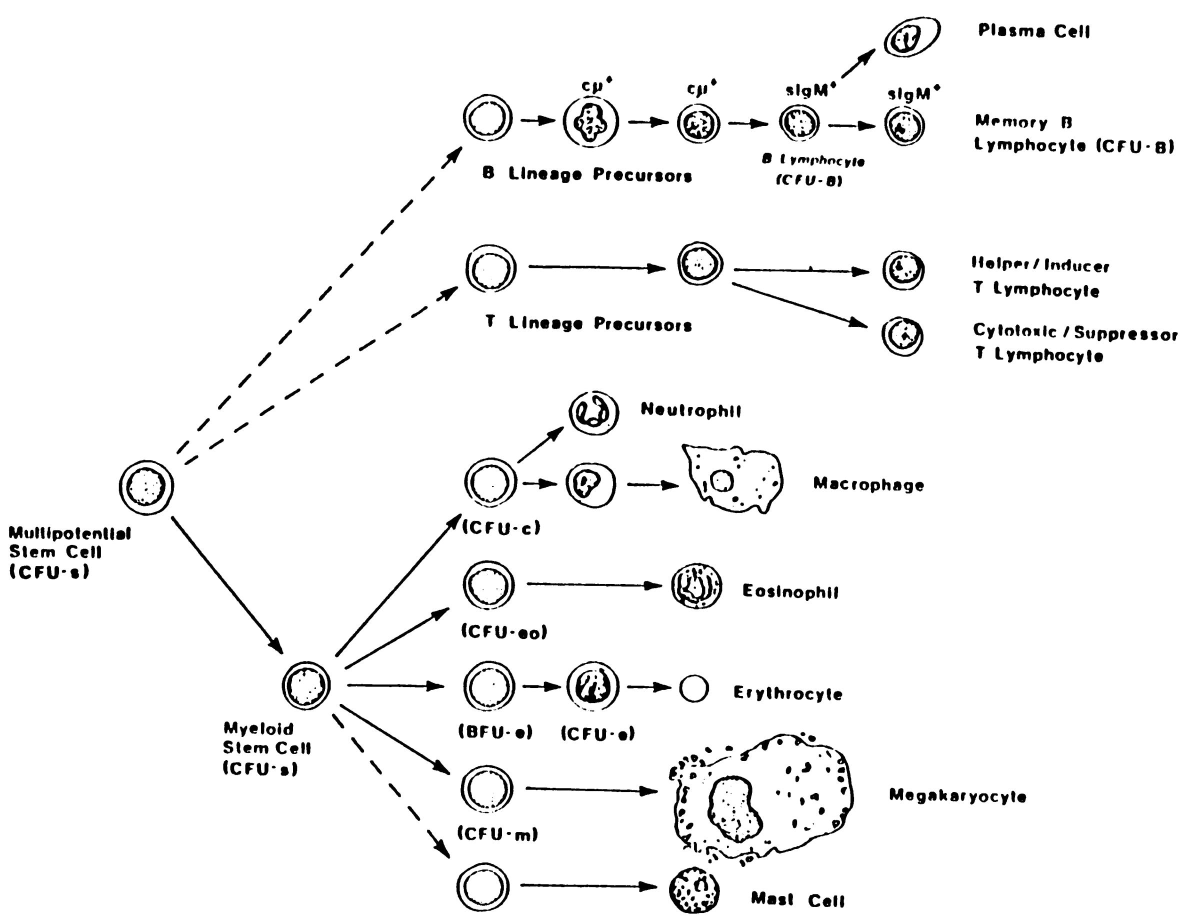
Apoptosis in B-lineage Cells

GC was shown to induce apoptosis in a neoplastic B-cell line (chronic lymphocytic leukemia of B-cell type, B-CLL) (McConkey *et al.*, 1991). Methyl-PD caused an increase in the cytosolic Ca^{2+} concentration followed by DNA fragmentation in B-CLL cells *in vitro*. Phorbol ester, cycloheximide (protein synthesis inhibitor) and a GcR antagonist all inhibited apoptosis in these cells, suggesting a similar mechanism to that of thymocytes (McConkey *et al.*, 1991). These data indicated that GC-induced apoptosis in B-CLL cells may be important pharmacologically; however, there was no indication from the literature that induction of apoptosis by endogenously produced GC could be an important regulator of normal B-cell lymphopoiesis during chronic stress. This dissertation addressed the question of whether or not GC can induce apoptosis in normal BM B-lineage cells.

Additionally, little was known about the role apoptosis may play in normal B-cell development. Rolink *et al.* (1991) recently determined that the loss of cells between the pre-B and immature B-cell stage of development (possibly due to a nonfunctional gene rearrangement) appeared to occur via apoptosis. Neiman *et al.* (1991) found that disruption of the microenvironment of developing B-cells in chicken bursa led to extensive cell death by apoptosis. These studies suggested that normal B-lineage cells possess the necessary apoptotic biochemical pathways and that apoptosis could play an important role in the normal development of B-lineage cells.

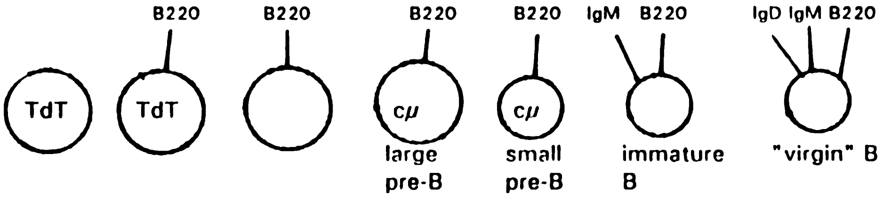
An important hypothesis put forward in this dissertation was that if the lymphopenia caused by chronic stress was due to impaired lymphopoiesis in the BM, it might be that the mechanism of depletion was via apoptosis. While there has been a profound interest in GC-induced apoptosis in the thymus over the last ten years, the literature is conspicuously devoid of studies addressing the possibility that GC may affect immature B-lineage cells in a similar way. One of the problems inherent in studying B-cell development was the heterogeneous nature of the BM. The assays for detecting apoptosis in thymocytes assumed a fairly homogeneous population of cells with a large proportion simultaneously induced to undergo apoptosis. Quantitative assays require isolation of DNA from a large number of purified cells and determination of the proportion which is fractionated by high-speed centrifugation, followed by electophoresis or quantitative assay of the low molecular weight fractions in the supernatant. Recently a method has been developed by William Telford and Louis King in our laboratory which has the potential for quantitating the proportion of apoptotic cells in a heterogeneous population (Telford *et al.*, 1991). Using a fluorescent DNA probe, propidium iodide, and flow cytometry it has been shown that cells undergoing apoptosis develop a "hypodiploid area" to the left of G_0/G_1 in a cell cycle profile termed the A_0 peak (Figure 1.9) (Telford *et al.*, 1991). Though others had seen a similar phenomenon using various fluorescent DNA dyes (Pechatnikov *et al.*, 1986; Compton & Cidlowski, 1988), Telford *et al.* (1991) correlated the A_0 peak with the appearance of internucleosomal DNA fragmentation on agarose gels and determined that the peak would disappear when exposed to inhibitors of macromolecular synthesis, GcR antagonists, and high zinc, all of which have been used routinely in the literature to inhibit apoptosis (Cohen & Duke, 1984; Compton & Cidlowski, 1986; McConkey *et al.*, 1990b). In addition, the A_0 peak was induced by both γ-irradiation and GC (Figure 1.9), classical inducers of apoptosis (Telford *et al.*, 1991; Sellins & Cohen, 1987; Wyllie, 1980). This method had tremendous potential in that it would facilitate the examination of apoptosis in BM B-lineage cells at different stages of development. Thus, subpopulations of BM cells were fluorescently labelled with antibodies to various cell surface markers and then DNA stained with propidium iodine. Examination of their cell cycle profiles and particularly the development of apoptotic regions were used extensively in this dissertation to document the effects of GC on BM B-lineage cells both *in vivo* and *in vitro*.

Figure 1.1 Immune cell lineages in murine bone marrow.



Plasma Cell
cμ+
cμ+
sIgM+
sIgM+
Memory B
Lymphocyte (CFU-B)
B Lineage Precursors
B Lymphocyte
(CFU-B)
Helper / Inducer
T Lymphocyte
T Lineage Precursors
Cytotoxic / Suppressor
T Lymphocyte
Neutrophil
Macrophage
(CFU-c)
Multipotential
Stem Cell
(CFU-s)
Eosinophil
(CFU-eo)
Erythrocyte
(BFU-e)
(CFU-e)
Myeloid
Stem Cell
(CFU-s)
Megakaryocyte
(CFU-m)
Mast Cell

Figure 1.2 Schematic representation of the stages of B-cell development in murine bone marrow.



Genes Rearranged		DJ_H		V_HDJ_H		V_LJ_L V_HDJ_H	
Size μm	9	10	11.5	>9	8	8	8
Percent of Total Bone Marrow	0.8%	3%	4%	5%	5%	7-15%	3-7%
Turnover Rate %/hr	5.1	9	13	15	Non-cycling		
Cells/day	3×10^6	5×10^6	36×10^6	35×10^6	17×10^6	16×10^6	

Figure 1.3 Heavy chain gene rearrangement in murine B-lineage cells.

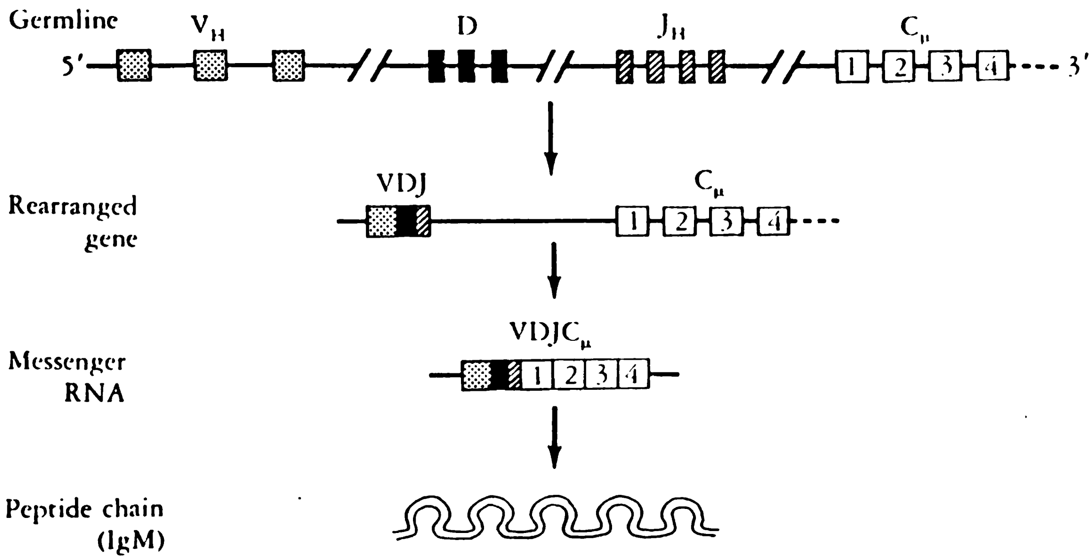
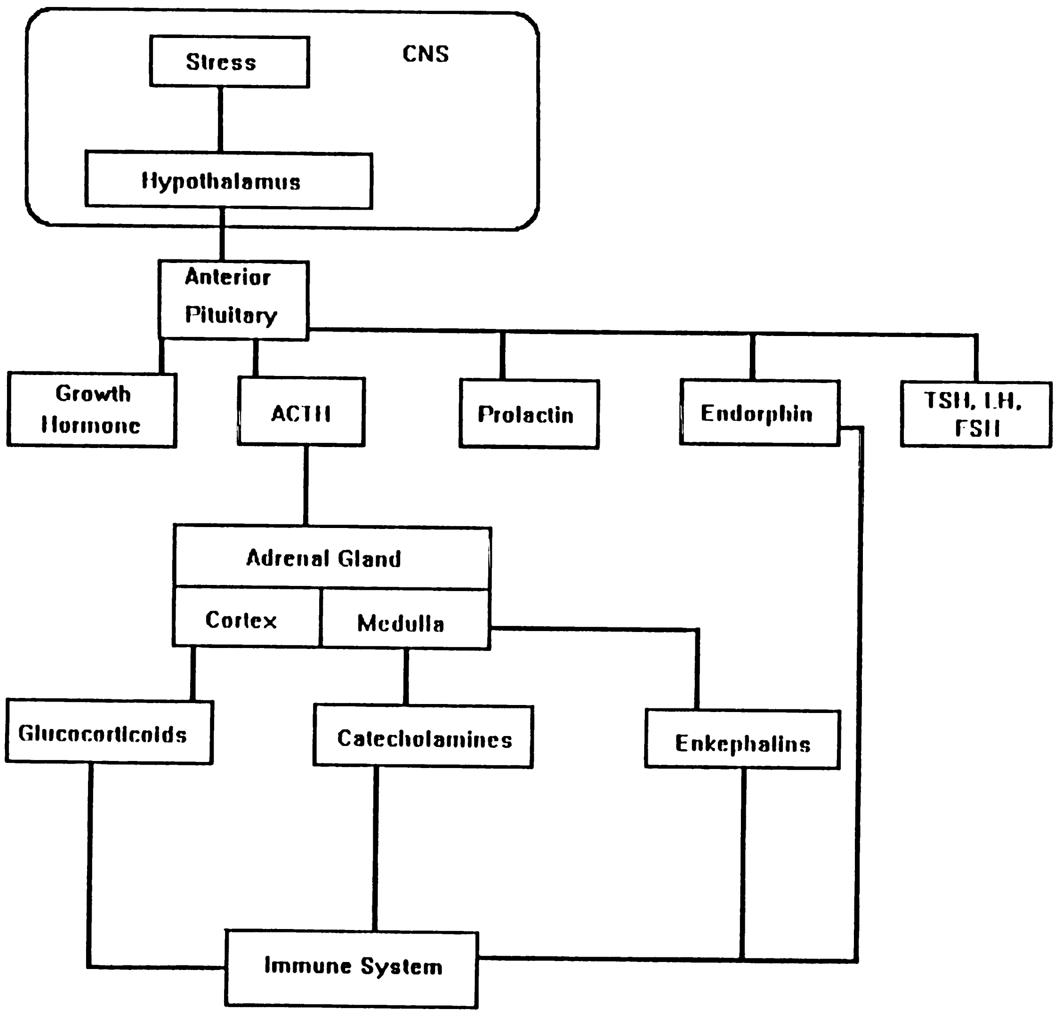
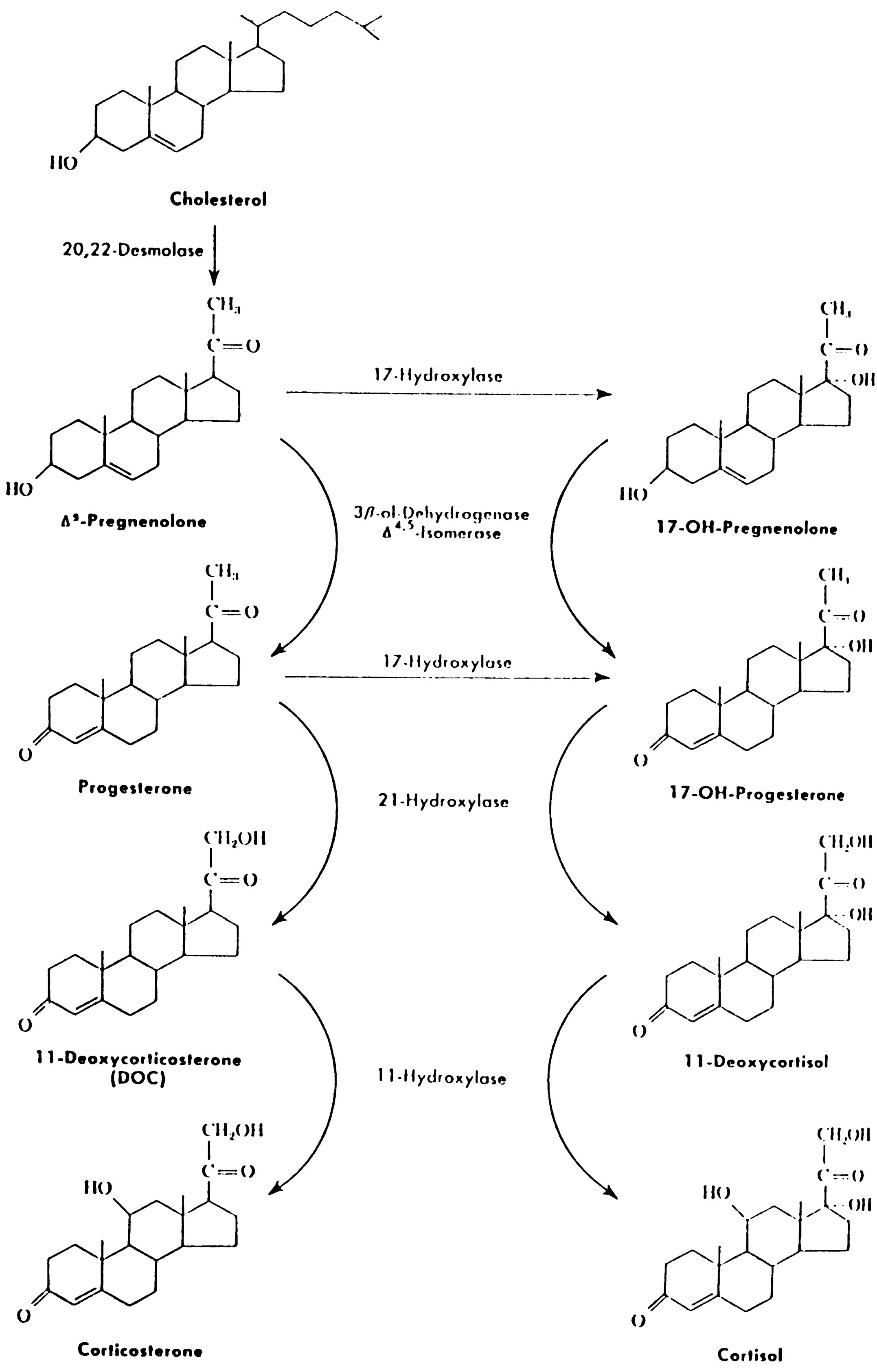

Germline
V_H
D
J_H
C_μ
5'
3'
1
2
3
4
VDJ
C_μ
Rearranged gene
1
2
3
4
$VDJC_\mu$
Messenger RNA
1
2
3
4
Peptide chain (IgM)

Figure 1.4 Schematic diagram of the hypothalamic-pituitary-adrenal cortex stress axis.



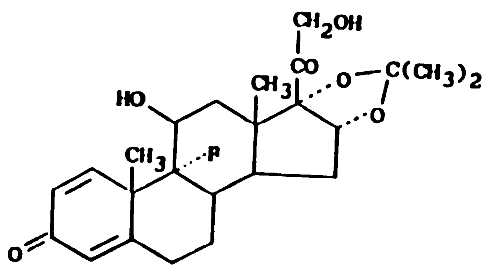
CNS
Stress
Hypothalamus
Anterior Pituitary
Growth Hormone
ACTH
Prolactin
Endorphin
TSH, LH, FSH
Adrenal Gland
Cortex
Medulla
Glucocorticoids
Catecholamines
Enkephalins
Immune System

Figure 1.5 Biochemical pathway of glucocorticoid synthesis in the adrenal cortex.

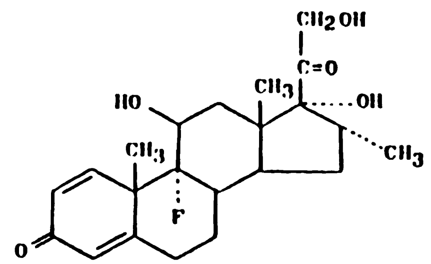


Cholesterol
20,22-Desmolase
Δ⁵-Pregnenolone
17-Hydroxylase
17-OH-Pregnenolone
3β-ol-Dehydrogenase
Δ^4,5-Isomerase
Progesterone
17-Hydroxylase
17-OH-Progesterone
21-Hydroxylase
11-Deoxycorticosterone
(DOC)
11-Deoxycortisol
11-Hydroxylase
Corticosterone
Cortisol

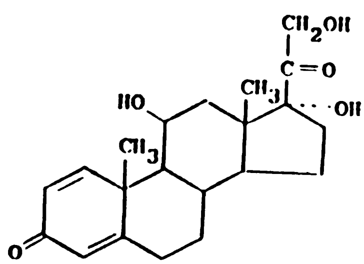
Figure 1.6 Structural comparisons of some commonly used synthetic glucocorticoids and a glucocorticoid receptor antagonist.



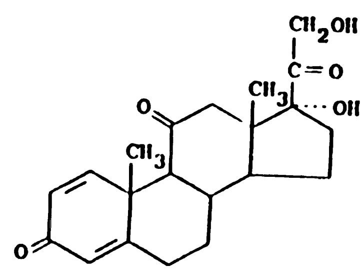
Triamcinolone
Acetonide



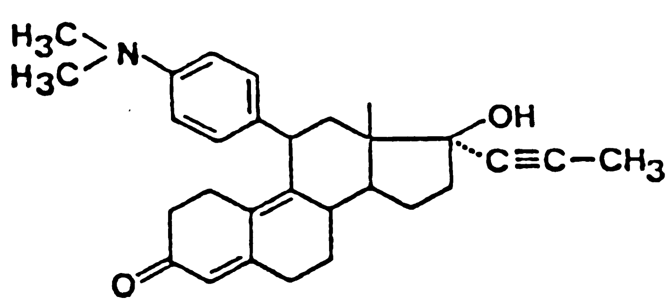
Dexamethasone



Prednisolone



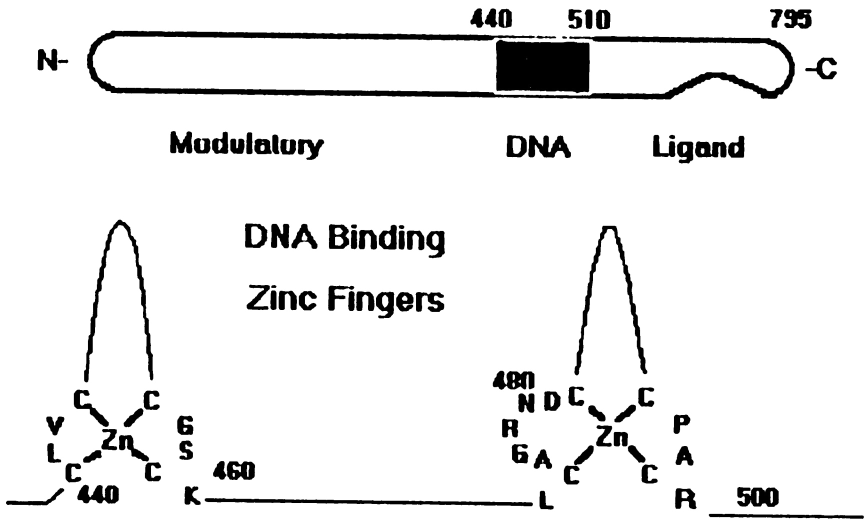
Prednisone



RU 38486

Figure 1.7 Schematic diagram of the structure of the glucocorticoid receptor. The domain structure is shown in the upper portion of the diagram along with the two zinc fingers. Also shown is the 15-mer consensus sequence of the glucocorticoid response element (GRE) found in the promoter region of hormone-sensitive genes.

Glucocorticoid Receptor



GRE Concensus Sequence

5' GGTACAnnnTGTTCT 3'

Figure 1.8 Diagram of nucleosomes and DNA linker regions. Endonucleases act in the linker regions causing fragments of approximately 200 base pair multiples.

a

b

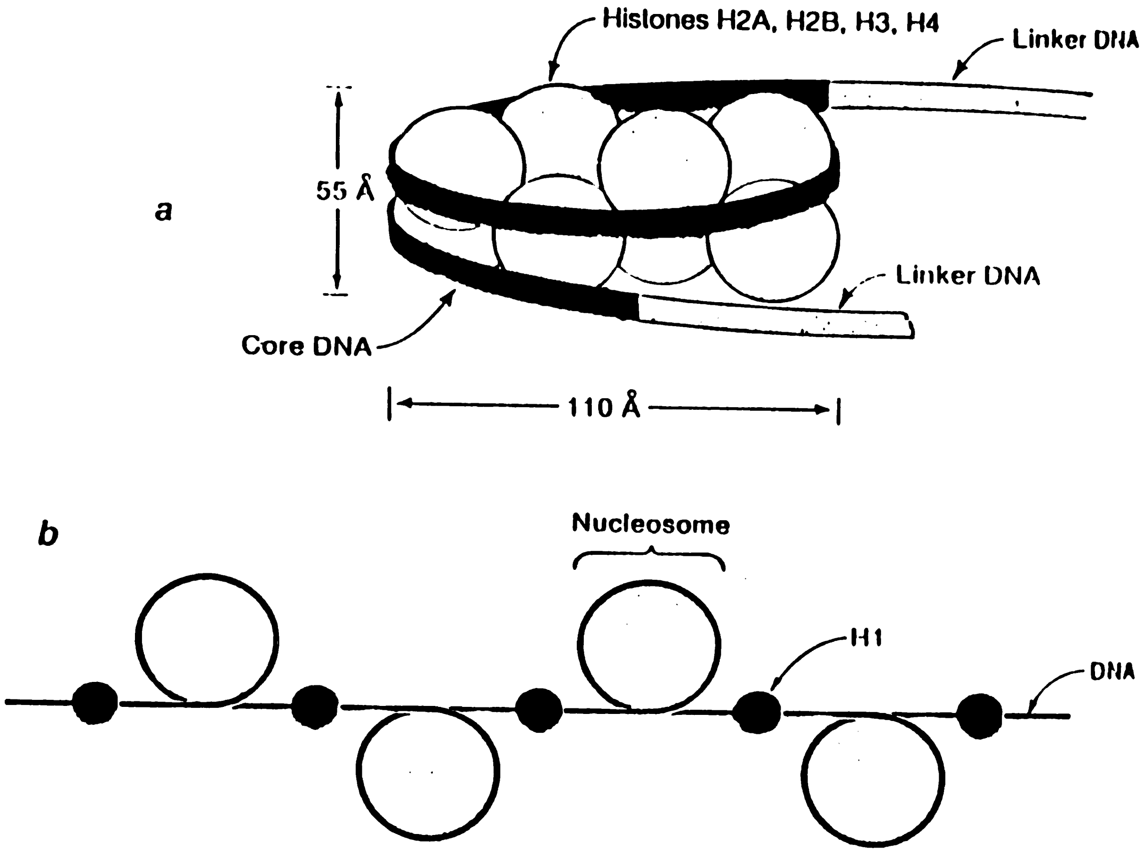
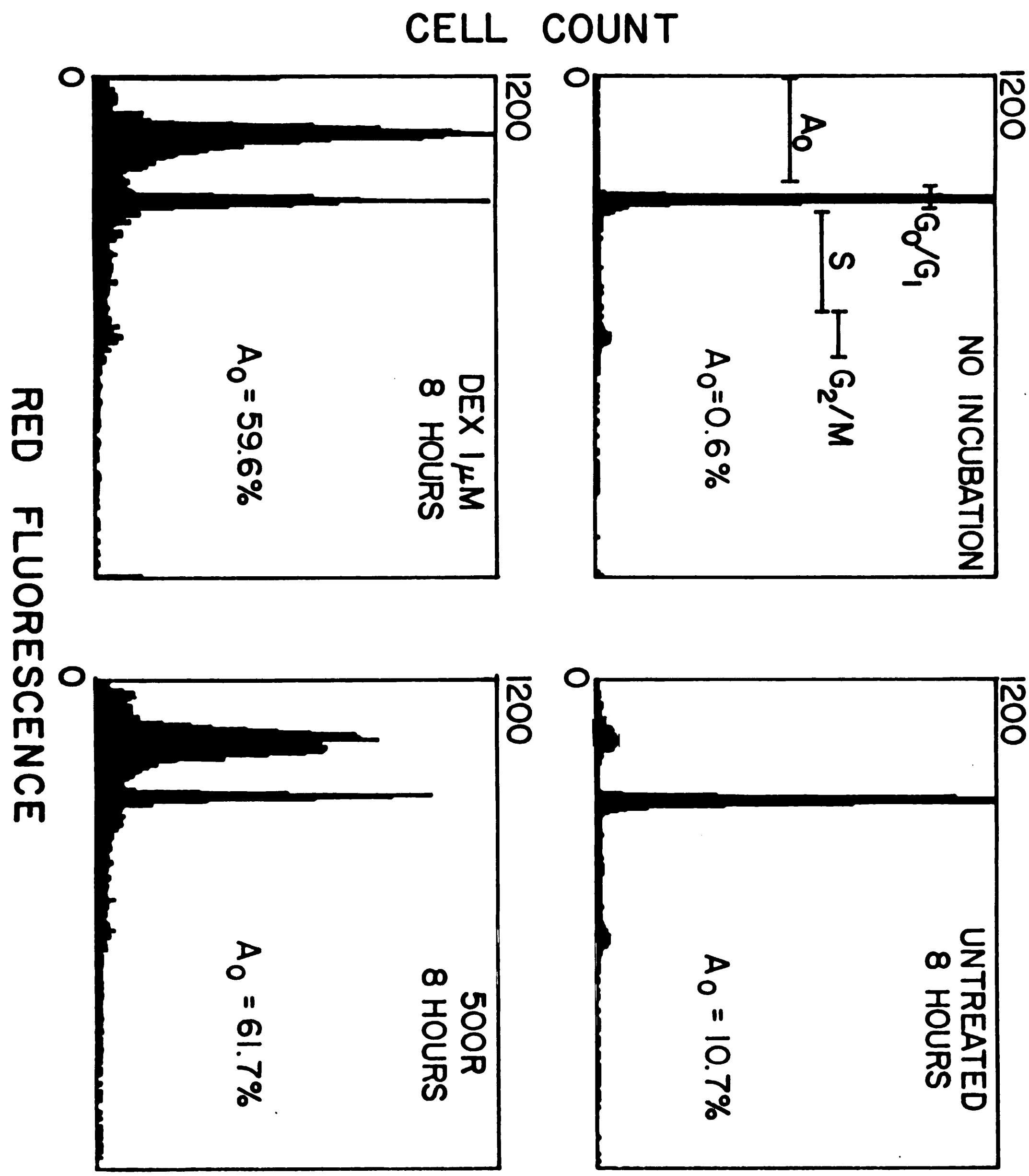

Histones H2A, H2B, H3, H4
Linker DNA
55 Å
Linker DNA
Core DNA
110 Å
Nucleosome
H1
DNA

Figure 1.9 Thymocyte cell cycle histogram showing apoptotic region. Thymocytes were incubated for 8 hours either with 1 μM dexamethasone or after irradiation and DNA stained with propidium iodide. DNA (red) fluorescence is expressed on the x-axis and cell count is on the y-axis. Areas relating to phases of the cell cycle are shown in the upper left panel. Cells which were "hypodiploid" appeared in a peak to the left of G_0/G_1 in an area called the A_0 region. These data were collected by W. Telford.


CELL COUNT
1200
0
NO INCUBATION
A_0
G_0/G_1
S
G_2/M
A_0=0.6%
UNTREATED
8 HOURS
A_0 = 10.7%
DEX 1 μM
8 HOURS
A_0 = 59.6%
500R
8 HOURS
A_0 = 61.7%
RED FLUORESCENCE

Chapter 2

Analysis of Glucocorticoid Induced Apoptosis in Murine B-Lineage Lymphocytes By Flow Cytometry

SUMMARY

A substantial proportion of murine bone marrow (BM) B220$^+$ and IgM$^+$ cells were induced to undergo apoptosis upon exposure to physiological levels of various glucocorticoids (GC) *in vitro*. Two-color flow cytometric analyses of the cell cycle indicated that a distinct subpopulation of cells formed to the left of G_0/G_1 in the hypodiploid region which has previously been termed A_0. The formation of the A_0 region was accompanied by an increase in cell density and internucleosomal DNA fragmentation indicative of programmed cell death. GC-induced apoptosis was dose-dependent and inhibited by the GC receptor antagonist RU 38486. The rate of entry of B220$^+$ and IgM$^+$ cells into apoptosis was similar with almost 90% of each cell type being apoptotic after 36 hours of exposure to GC. In contrast to previous reports, these results demonstrated that freshly isolated B-lineage cells of the bone marrow can undergo apoptosis upon exposure to GC. These data also suggest that apoptosis may play a role in B-cell development and regulation of lymphoiesis. The high degree of sensitivity of flow cytometric cell cycle analysis for quantitating the degree of apoptosis in subsets of cells within a heterogenous population indicate that it is much superior to gel electrophoresis of DNA for detecting and quantitating programmed cell death.

INTRODUCTION

Apoptosis, or programmed cell death, is observed in a diverse array of cellular systems (Wyllie *et al.*, 1980b). Its role in immune function and regulation is under particularly intense scrutiny. Murine thymocytes can be induced to undergo programmed cell death in response to a wide variety of agents, including GC (Wyllie, 1980; Umansky *et al.*, 1981; Cohen & Duke, 1984; Compton & Cidlowski, 1986). Programmed cell death is also thought to play an active role in target cell destruction by cytotoxic T-lymphocytes and natural killer cells (Ucker, 1987; Hasegawa & Bonavida, 1989). Antibodies to the CD3 component of the T-cell receptor have been shown to induce apoptosis in thymocytes leading to speculation regarding the role of programmed cell death in clonal deletion of autoreactive T-cells (Smith *et al.*, 1989; MacDonald & Lees, 1990; Ucker *et al.*, 1989).

More recently, considerable attention has been directed at induction of apoptosis in hematopoietic precursor cells and early lineage B-cells since apoptosis has been shown to be prevalent in other immature cells such as thymocytes. Factor deprivation has been found to induce apotosis in hematopoietic precursor cell lines and immortalized pre-B lymphocytes (Williams *et al.*, 1990; Rodriguez-Tarduchy *et al.*, 1990; Sabourin & Hawley, 1990). Antibodies to surface IgM have been found to induce apoptosis in the immature B-cell lymphoma WEHI 231 cell line suggesting that, as for thymocytes, apoptosis may play a role in deletion of autoreactive B-cells (Benhamou *et al.*, 1990; Page & DeFranco, 1990). GC have been observed to initiate programmed cell death in B-cell type chronic lymphocytic leukemia cells (McConkey *et al.*, 1991). However, there was no evidence from the literature that GC would induce apoptosis in normal B-lineage cells as has been described repeatedly for thymocytes. This was an important question since plasma corticosterone concentrations reach levels during chronic stress which have been shown to induce apoptosis in thymocytes *in vitro* (Telford *et al.*, 1991; Cohen & Duke, 1984).

One of the problems with studying apoptosis in developing B-cells is the heterogeneous nature of the bone marrow. Cohen and Duke (1984) reported that unlike thymocytes, whole bone marrow did not have an endogenous endonuclease since DNA fragmentation was not observed upon exposure of nuclei to calcium and magnesium. However, they made no effort to examine subpopulations within the BM. The majority of the studies investigating apoptosis in B-lineage cells were limited to transformed or immortalized cell lines and neoplastic tissues, primarily because of limitations imposed by present techniques for evaluating apoptosis which are based on analysis of cell lysates for detecting fragmented DNA or ultrastructural evaluation by electron microscopy. The requirement of these methods for large numbers of cells with a high degree of homogeneity made the evaluation of normal hematopoietic progenitor cells or B-lineage lymphocytes in BM virtually impossible due to the heterogeity of the tissue, the relatively low percentage of any given cell type, and the possibility that apoptotic pathways may vary among different cell types.

It has been previously observed that cells undergoing apoptosis-associated DNA fragmentation form a distinct quantifiable cell cycle subpopulation after staining with an intercalating DNA dye such as acridine orange or propidium iodide, thus allowing detection of apoptosis in intact cells by flow cytometry (Compton *et al.*, 1988; Telford *et al.*, 1991; Pechatnikov *et al.*, 1986). This technique was used in conjunction with fluorescent immunophenotyping to identify apoptotic subpopulations of B-lineage cells in primary cultures of mouse bone marrow as will be discussed. The data presented in this chapter show that $B220^+$, $sIgM^+$, and $sIgD^+$ lymphocytes in murine BM undergo apoptosis following exposure to physiological concentrations (0.1-1.0 μM) of GC *in vitro* and that this apoptosis is inhibited by the addition of the GC receptor antagonist RU 38486. These findings suggested that physiological concentrations of GC may be an important regulator of lymphopoiesis during chronic stress by the induction of apoptosis in developing B-lineage cells.

METHODS

Short Term Bone Marrow Cell Culture with Gluococorticoids

Bone marrow from A/J mice (6-12 weeks old, The Jackson Laboratory, Bar Harbor, ME) was flushed from femurs and tibias and suspended by aspiration into phosphate buffered saline, pH 7.4 (PBS), containing 2% fetal calf serum FCS). Red blood cells were removed by centrifugation at 500Xg for 10 minutes through a density gradient (Histopaque 1083, Sigma Chemical Co., St. Louis, MO) and leukocytes were recovered at the interface. Cells were washed twice (by centrifugation at 400Xg for 5 minutes), counted, and suspended at a concentration of 10^6 cells/ml in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD) supplemented with 2mM L-glutamine, $5x10^{-5}$ M mercaptoethanol, 5% FCS (M.A. Bioproducts) and 0.5% globulin free bovine serum albumin (Sigma). Aliquots of cells (1 ml) were pipetted into 24 well plates and incubated in a humidified chamber under an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 at 37°C. Glucocorticoids (Sigma) and RU 38486 (Roussel-Uclaf, Romainville, France) were dissolved in 95% ethanol at a concentration of 1 mg/ml, diluted in RPMI-1640, and added to cells so that the cultures never exceeded 0.1% ethanol.

Phenotypic Staining of Apoptotic Cells for Flow Cytometric Analysis

The induction of apoptosis in bone marrow B-lineage cells after culture with varying concentrations and types of GC was evaluated by phenotypic staining and cell cycle analysis using a modification of the technique of Telford *et al.* (1991) At time points up to 48 hours in culture, cells were harvested and determined to be greater than 80% viable by trypan blue exclusion. Aliquots of $2x10^6$ cells were incubated with 10% rat serum to reduce nonspecific binding and labelled with 2.0 μg fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgD (The Binding Site, Birmingham, England), 1.5 μg anti-B220 (isolated from RA3.6B2 cell culture supernatants and biotinylated), or 0.9 μg of biotin-conjugated goat anti-mouse IgM (Cappel, Malvern, PA). Cells were incubated with antibodies for 30 minutes at 4°C in PBS with 4% heat-inactivated FCS and 0.15% NaN_3 and then washed in ice cold buffer. Biotinylated antibodies were further incubated for 30 minutes with 3 μg avidin-FITC (Vector, Burlingame, CA). Cells were suspended in PBS with 50% heat-inactivated FCS and fixed by dropwise addition of ice cold 70% ethanol to a final concentration of 50% ethanol (Zarbo *et al.*, 1989). After storage overnight at 4°C, the cells were washed with PBS to remove fixative, stained in 1 ml of a propidium iodide (PI) staining reagent (PBS pH 7.4 with 0.05 mg/ml RNase A, Boehringer Mannheim Biochemicals, Indianapolis, IN, at 50 units/mg and 50 μg/ml PI, Calbiochem, San Diego, CA) at room temperature for 1 hour, and placed on ice until analysis on the same day.

Flow Cytometric Analysis of Apoptotic Cells

Quantitation of the proportion of B-lineage cells undergoing apoptosis was performed by cell cycle analysis using an Ortho Diagnostics Cytofluorograph 50-H/Intel 80386 computer and Acqcyte software (Phoenix Flow Systems, Inc., San Diego, CA). FITC and PI were excited with the 488 nm line of an argon laser with emission detection at 530±15 nm and 620-700 nm, respectively. PI emission in the FITC detection range was corrected by electronic compensation. FITC-positive cells were examined for cell cycle distribution using a 3-step gating procedure which eliminated cell debris, cell doublets, and nonspecific antibody binding. Dexamethasone (DX)-treated thymocytes were used as a positive apoptotic control since they have been shown to develop an A_0 peak to the left of the G_0/G_1 peak which corresponds to cells undergoing DNA fragmentation (Telford *et al.*, 1991). The coefficient of variation for the G_0/G_1 peak was typically less than 3.5 for all samples.

Identification of Internucleosomal DNA Fragmentation in Bone Marrow B-cells Enriched by "Panning"

To confirm that those cells found in the A_0 region were undergoing internucleosomal DNA fragmentation, bone marrow cells treated with DX were enriched for B220 positive cells using a modification of the "panning" method of Wysocki and Sato (Wysocki & Sato, 1978) and DNA isolated for analysis using gel electrophoresis. After 5 hours in culture, bone marrow cells were removed from flasks and incubated at 4°C for 1 hour on plates coated with B220 from ascites fluid raised in CAF_1 mice inoculated with RA3.3A1 cells. Nonadherent cells were flushed from the plates with PBS and adherent cells were lysed with 20 mM Tris pH 8.5, 10 mM EDTA disodium salt, 1% SDS, and 200 μg/ml proteinase K. Purity of $B220^+$ cells was routinely greater than 80% after "panning" (greater detail is given in chapter 5). DNA was extracted from cell lysates using phenol:chloroform:isoamyl alcohol (49:49:2), precipitated with 70% ethanol at -70°C, dried by vacuum centrifugation, and resuspended in 100 μl 20 mM Tris pH 8.5, 10mM EDTA (Wallace, 1987). DNA concentrations were determined using a Hoechst 33258 assay (Labarca & Paigen, 1980) so that equal amounts of DNA would be loaded into each lane for electrophoresis. For this purpose, part of the DNA samples were diluted to a final volume of 1 ml into a high salt buffer of 0.05 M $NaPO_4$, 2.0 M NaCl, pH 7.4 containing 2 mM EDTA and 20 μl of Hoechst 33258 (50 μg/ml) added. Fluorescence was excited at a wavelength of 356 nm and emission was detected at 460 nm on a Perkin Elmer Spectrofluorimeter. Salmon sperm DNA was used as a standard and gave a linear response over a range of 0.5-5.0 μg (Figure 2.1).

To determine the degree of fragmentation, DNA samples (5 μg/lane) were electrophoresed on 1.5% horizontal agarose gels with bromphenol blue tracking dye. Gels were run at 45 V for

6 hours submerged in 40 mM Tris, 20 mM acetic acid, 1 mM EDTA disodium salt, stained with 5 μg/ml ethidium bromide and photographed under UV light. A HindIII restriction digest of lambda phage DNA (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) was used as a molecular size marker.

RESULTS

Identification of Apoptotic Subpopulations in Mouse Bone Marrow by Phenotype-Gated Cell Cycle Analysis

To determine if developing murine B-cells undergo GC-induced apoptosis, primary cultures of mouse BM were incubated for up to 48 hours with physiological levels of DX. The cells were labelled with fluorescent antibodies against the B-lineage lymphocyte-associated surface markers B220 or IgM and subjected to flow cytometric propidium iodide cell cycle analysis using methods adapted from Telford, *et al.* (1991). Analysis of light scatter characteristics of cells bearing the fluorescent markers by flow cytometry was used to provide information regarding the size (forward scatter) and granular content of cells (90° or side scatter) which had been treated with 0.1 μM DX for 16 hours (Figure 2.2). A significant proportion (58.6%) of B220$^+$ cells in the DX treated BM underwent a downward shift in size after exposure to DX (Figure 2.2). There was a similar shift in IgM$^+$ cells after DX treatment (data not shown). These data were consistent with the morphological characteristics noted for thymocytes undergoing apoptosis which have been shown to increase in density and decrease in size (Wyllie & Morris, 1982).

Propidium iodide is a fluorescent dye which binds to DNA in stoichiometric amounts. Cells which are in the G_2/M phase of the cell cycle with duplicated DNA exhibit twice as much fluorescence when analyzed by flow cytometry as cells which are in G_0/G_1 phase and have single copies of DNA. Cell cycle histograms derived for the DX (1 μM) treated B220$^+$ and IgM$^+$ subpopulations demonstrated a distinct, well-defined accumulation of cells below the G_0/G_1 region (Figure 2.3) with the same relative position and discrete nature as the A_0 region previously observed for apoptotic thymocytes (Telford *et al.*, 1991). In addition to shifting in size, cells falling into the A_0 region exhibit lower PI fluorescence than normal cells in the G_0/G_1 phase. The percentage of cells in the A_0 region was over 45% of all B220$^+$ cells and 50% of all IgM$^+$ cells after 12 hours of treatment with DX. Fewer than 18% of the B220$^+$ and the IgM$^+$ cells in untreated samples were found in the A_0 region after the same incubation period (Figure 2.3). Only about 1% of freshly isolated B-lineage cells were found in the A_0 region. Note from Table 1 that the viability of these cells, as assessed by trypan blue exclusion, was greater than 85% after 16 hours of 1 μM DX treatment (Table 2.1). This finding is consistent with reports in the literature which indicated that apoptotic cells undergo DNA fragmentation before they show any loss in ability to exclude vital dyes (Compton & Cidlowski, 1986).

The high proportion of B220$^+$ and IgM$^+$ cells in the A_0 region suggested that lymphocytes expressing these phenotypic markers underwent apoptosis following exposure to glucocorticoids and constituted a significant proportion of all cells undergoing apoptosis in whole BM. Indeed, of the total BM cells found below G_0/G_1, 40-45% were B220$^+$ cells 12 hours after exposure to DX treatment (Table 2.2). IgM bearing cells accounted for about 25% of the total BM cells undergoing apoptosis (Table 2.2). Though the total proportion of cells in the A_0 region of untreated BM was low (10%), 20% and 28% of these cells were also IgM$^+$ and B220$^+$, respectively (Table 2.2). Collectively, the data suggest that BM B-lineage cells may be as sensitive to DX as immature thymocytes.

The kinetics of DX-induced accumulation of B220$^+$ and IgM$^+$ lymphocytes in the A_0 region were evaluated over 10 hours (Figure 2.4). The proportion of B220$^+$ and IgM$^+$ cells in the A_0 region increased nearly linearly over 10 hours of exposure to 1 μM DX. The rate of accumulation of cells in the A_0 region was slightly greater in the IgM$^+$ than the B220$^+$ cells. While at 2 and 3 hours the proportions of IgM$^+$ and B220$^+$ cells in the A_0 region were approximately the same (3% at 2 hours and 7% at 3 hours); at 10 hours, greater than 20% of the IgM$^+$ cells had accumulated in the A_0 region compared to about 15% of B220$^+$ cells. Since the proportion of B-lineage BM cells in the A_0 region did not plateau at 10 hours, cells incubated with 1 μM DX were evaluated up to 48 hours. Interestingly, both B220$^+$ and IgM$^+$ continued to accumulate until 90% were in the A_0 region after 36 hours of incubation with DX (Figure 2.5). This persistent increase in cells accumulating in the

A_0 region was accompanied by a steady decrease in the proportion of B-lineage cells. At 36 hours there were only 5.2% and 3.5% $B220^+$ and IgM^+ cells remaining in the cultures. Surprisingly, IgD^+ cells also accumulated in the A_0 region but only after a significant lag. Whereas around 40% of $B220^+$ and IgM^+ cells were found in the A_0 region at 12 hours, only slightly over 20% of IgD^+ cells appeared in this region. However, by 36 hours 90% of the IgD^+ cells were in the A_0 region (Figure 2.5). These data suggest that though the more mature IgD^+ B-cells of the BM were initially more resistant *in vitro* than their immature counterparts, eventually they also succumbed to the effects of DX.

Concentrations from 1μM to 0.001 μM of DX and the natural GC, corticosterone (CS) and cortisol (HC) were evaluated to compare their ability to induce the accumulation of $B220^+$ and IgM^+ bone marrow B lymphocytes in the A_0 region after 8 hours in culture (Figure 2.6). Effects of these glucocorticoids were all dose-dependent, with minimum effective doses of 0.1 μM for CS and HC and 0.01 μM for DX. The percentage of apoptotic cells plateaued for concentrations above these doses. This is consistent with the dose dependency observed by Telford *et al.* (1991) for thymocytes after 8 hours exposure to DX and HC. These data suggest a higher potency for the synthetic DX than for the natural GC, HC and CS, which are predominant in humans and mice, respectively. Nevertheless, it should be noted that the minimum effective doses for HC and

CS are within the physiological range of plasma levels found during such chronic stresses as malnutrition, trauma, and burns (DePasquale-Jardieu & Fraker, 1980; Kagan *et al.*, 1989; Hamanaka *et al.*, 1970).

RU 38486, a GC receptor antagonist (Moguilewsky & Philibert, 1984) known to inhibit GC-induced apoptosis in mouse thymocytes (Compton & Cidlowski, 1986; Schwartzman & Cidlowski, 1991), effectively inhibited 0.1 μM DX induced BM B-lineage cell apoptosis to near baseline levels over 16 hours (Figure 2.7). Zinc, which at high concentrations of 500 μM also inhibits thymocyte apoptosis (Cohen & Duke, 1984; Telford *et al.*, 1991), was found to protect B220$^+$ and IgM$^+$ B-lineage lymphocytes from the apoptotic effects of DX (not shown, data collected by W. Telford). Cell viabilities were improved when RU 38486 was used to inhibit apoptosis (Table 1). RU 38486 had no adverse effects on BM cells when added to cultures alone (data not shown).

Verification of Apoptosis in Bone Marrow B-lineage Cells by Analysis of Whole Cell Lysates for Fragmented DNA

Analysis of induction of apoptosis with DX was used to demonstrate the superior sensitivity of flow cytometry for the detection of apoptosis and to verify that the presence of internucleosomal DNA fragmentation corresponded to appearance of cells in the A_0 region of the cell cycle. Therefore, analyses were carried out at an early time point when only a modest number of apoptotic B-cells were present. Whole BM incubated with 1 μM DX was separated after 5 hours into B220$^+$ and B220$^-$ cell fractions by panning over RA3.3A1. The resulting adherent and nonadherent fractions were lysed, and purified DNA extracts were subjected to agarose electrophoresis to determine the presence of internucleosomal DNA fragmentation. Purity of B220$^+$ cells was routinely greater than 80%. B220$^+$ and, to a lesser extent, B220$^-$ cell fractions both demonstrated internucleosomal DNA fragmentation (as did thymocytes) consistent with apoptosis after treatment with GC (Figure 2.8). Analysis of 1 μM DX treated whole BM by cell lysates and DNA gel electrophoresis was deceptive in that, unlike thymocytes, there was no visually apparent endonuclease activity present after 5 hours (lane 7, Figure 2.8). This was consistent with previous reports in the literature where no fragmentation of DNA was noted in whole BM (Cohen & Duke, 1984). However, fractionation of the bone marrow did indicate that B220$^+$ cells were undergoing internucleosomal DNA fragmentation (lane 8, Figure 2.8). Concurrent cell cycle analysis of BM at the same 5-hour time point indicated that 18.0% of the DX treated B220$^+$ cells (see lane 8, Figure 2.8) were in the A_0 region while only 7.1% of total BM was apoptotic when analyzed by flow cytometry (data not shown) being analogous to that shown in Figure 2.3. Since DNA fragmentation was not readily detectable in DX-treated whole BM cell lysates, it is apparent that the sensitivity of the method was not adequate for detecting apoptosis in small subpopulations of heterogeneous BM cells. Conversely, two-color flow cytometric cell cycle analysis was able to detect apoptosis in small subpopulatins of DX-treated cells; it being a significantly more sensitive method for detecting apoptosis. Furthermore, the presence of internucleosomal DNA fragmentation in the cell fractions correlated with the presence of events in the A_0 region for $B220^+$ cells in mouse BM.

DISCUSSION

Physiological concentrations of GC have been found to rapidly induce apoptosis in a significant proportion of $B220^+$, IgM^+, and IgD^+ lymphocytes *in vitro* using murine BM. Their presence in the A_0 region of the cell cycle several hours prior to a significant loss in viability was noted due to their shift in size and DNA staining capacity. GC-induced apoptosis was inhibited by the GC receptor antagonist RU 38486 suggesting mediation through the classical GC receptor (Moguilewsky & Philibert, 1984). The presence of cells in the A_0 region was consistent with the presence of apoptosis-associated internucleosomal DNA fragmentation in fractionated mouse BM. Further, flow cytometric cell cycle analysis was demonstrated to be a superior method for quantitating the proportion of apoptotic cells in small subpopulations of the heterogeneous BM cells.

The requirements for inducing and inhibiting apoptosis in mouse B-lineage lymphocytes, as well as the resulting changes in cell volume (Wyllie & Morris, 1982), chromatin stainability (Telford *et al.*, 1991; Pechatnikov *et al.*, 1986), and dose-response data (Cohen & Duke, 1984; Telford *et al.*, 1991) for several glucocorticoids are clearly consistent with results previously obtained for apoptosis in mouse thymocytes. Programmed cell death in mouse B-lineage lymphocytes and immature thymocytes may thus proceed by pathways with common characteristics. Further, the developing and immature

B-lineage cells of the bone marrow appear to be as sensitive to glucocorticoids as are immature thymocytes. These ideas are consistent with the notion that apoptosis may be a pervasive regulatory process in the immune system.

The rate of GC induction of apoptosis was fairly rapid with significant numbers of B-lineage cells appearing in the A_0 region of the cell cycle within 3-4 hours. The proportion of IgM^+ cells which appeared in the A_0 region was slightly higher than $B220^+$ cells early in the culture period; however, after 16 hours the proportion of apoptotic cells was similar between the two cell types. There was no indication that a GC-resistant population of B-lineage cells existed in the BM since the IgM^+, IgD^+, and $B220^+$ cells remaining after 48 hours of culture with DX were greater than 90% apoptotic. Interestingly, IgD^+ BM cells became apoptotic here after a lag of around 12 hours. This was unexpected since IgD^+ is acquired late in development and these cells are considered to be fairly mature (Aspinall & Owen, 1983; Lala *et al.*, 1979). It has generally been thought that immature cells are much more sensitive to GC than mature cells since thymic cortical cells are preferentially deleted by GC, compared to more mature medullary cells (Weissman, 1973). Apparently, even "mature" cells can be induced to undergo apoptosis under the proper conditions.

Previous studies with mouse bone marrow have demonstrated a consistent pattern of pre-B lymphocyte deletion during normal B-lineage differentiation (Opstelten & Osmond, 1983) while early pre-B-cell clones possessing the capability of developing IgM have been shown to undergo apoptosis during this transition (Rolink *et al*., 1991; Ales-Martinez *et al*., 1991). In fact, the data presented here indicate that there is a low level of apoptosis which occurs in cultured B-lineage cells without further induction by GC. The demonstration that B220$^+$ and IgM$^+$ B-lineage lymphocytes in fresh mouse BM are capable of undergoing apoptosis suggests that programmed cell death may be an underlying mechanism in the deletion of B-lineage lymphocytes, in these and other systems, by a pathway with characteristics similar to those of thymic deletion. The recent work involving anti-IgM induction of apoptosis in WEHI 231 B-cell lymphomas (Benhamou *et al*., 1990), interleukin-induced inhibition of apoptosis in B-cell hybridomas (Rodriguez-Tarduchy *et al*., 1990; Liu *et al*., 1991), and the role of *bcl-2* and Epstein-Barr virus latent protein expression (Hockenbery *et al*., 1990; Gregory *et al*., 1991) in suppression of apoptosis in human B cells all clearly suggest that programmed cell death may play a pervasive role in the regulation of B-lineage lymphocyte differentiation and function.

Although B220$^+$ and IgM$^+$ lymphocytes make up a significant proportion of the cells undergoing apoptosis in mouse bone marrow, they are by no means the only cells to do so. The identity of the B220$^-$ BM cells undergoing apoptosis is not yet defined; however, a component of it is likely to represent cells composing the overall microenvironment necessary for the regulation of lymphocyte progenitor survival and differentiation. The observation that growth factor deprivation can induce apoptosis in haematopoietic progenitors (Williams *et al.*, 1990) and pre-B lymphocytes (Hockenbery *et al.*, 1990) suggests that damage to the BM microenvironment, and the resulting disruption of growth factor availability, may be contributing factors in B-lineage apoptosis. This is a particularly intriguing idea in light of the recent finding that the IL-7 receptor gene in murine pre-B cells has potential GREs upstream from the promoter (Pleiman *et al.*, 1991). IL-7 appears to essential for survival of pre-B cells (Era *et al.*, 1991; Kincade *et al.*, 1989), and down regulation of the IL-7 receptor could have deleterious effects on lymphopoiesis. The role that apoptosis may play in perturbation of the BM microenvironment and the resulting effects on B-lineage lymphocyte apoptosis complex issues which currently is being investigated.

B-lineage lymphocytes show considerable sensitivity to GC at early and late stages of differentiation with pharmacological doses being reported to cause depletion of murine BM $c\mu^+$ (Ku & Owen, 1986) and TdT^+ cells (Vines *et al.*, 1980) and rat BM sIg^+ cells (Sabelle *et al.*, 1987). Unfortunately, all of these studies used either DX or large (15 mg/mouse) (Ku & Owen, 1986) doses of HC. It is evident from the results presented in this chapter that the depletion of BM B-lineage cells by GC observed in the above *in vivo* studies may have been due to apoptosis. Though, in the current investigation, BM cells were exposed to GC *in vitro*, the data demonstrated for the first time that GC could induce BM B-lineage cells to undergo apoptosis at physiological levels. However, the effects of exposure of these cells to natural GC *in vivo* at concentrations reported during chronic stress remains unknown, but is addressed in a subsequent chapter.

Table 2.1 Viability of bone marrow cells after treatment with dexamethasone

Treatment	Percent Viability	
	8 hours	16 hours
Untreated	9.15	86.5
DX*	86.7	84.5
DX + Ru 38486†	89.0	90.7

*1 μM dexamethasone (DX)

†0.1 μM DX + 1 μM RU 38486

Bone marrow cells were incubated for up to 16 hours with the indicated compounds. Cell viability was determined by trypan blue exclusion prior to phenotypic labelling and cell cycle analysis. Data are representative of 4 separate experiments.

Table 2.2 Proportion of total apoptotic cells of whole bone marrow which were phenotype positive for B-cell markers

	Total Apoptotic Cells	Percent of All Apoptotic* Cells	
Treatment	Whole BM	B220$^+$	IgM$^+$
Untreated	9.6%	27.8%	19.9%
1 μM DX	14.2%	39.1%	24.4%

*Proportion of apoptotic cells in whole bone marrow which were phenotype positive

Bone marrow cells were cultured for 12 hours with or without dexamethasone (1 μM). Cells were phenotypically labeled, fixed in ethanol, and stained with propidium iodide for cell cycle analysis. Data are representative of 4 separate experiments.

Figure 2.1 Standard curve for Hoechst 33258 assay. Known amounts of salmon sperm DNA were incubated with 1 μg Hoechst 33258 and fluorescence excited at 356 nm with emission detected at 460 nm. The concentrations of DNA in the samples isolated from enriched bone marrow B-cells were determined from the standard curve so that equal amounts of DNA were applied to each lane of the agarose gels. Data are representative of two different experiments.

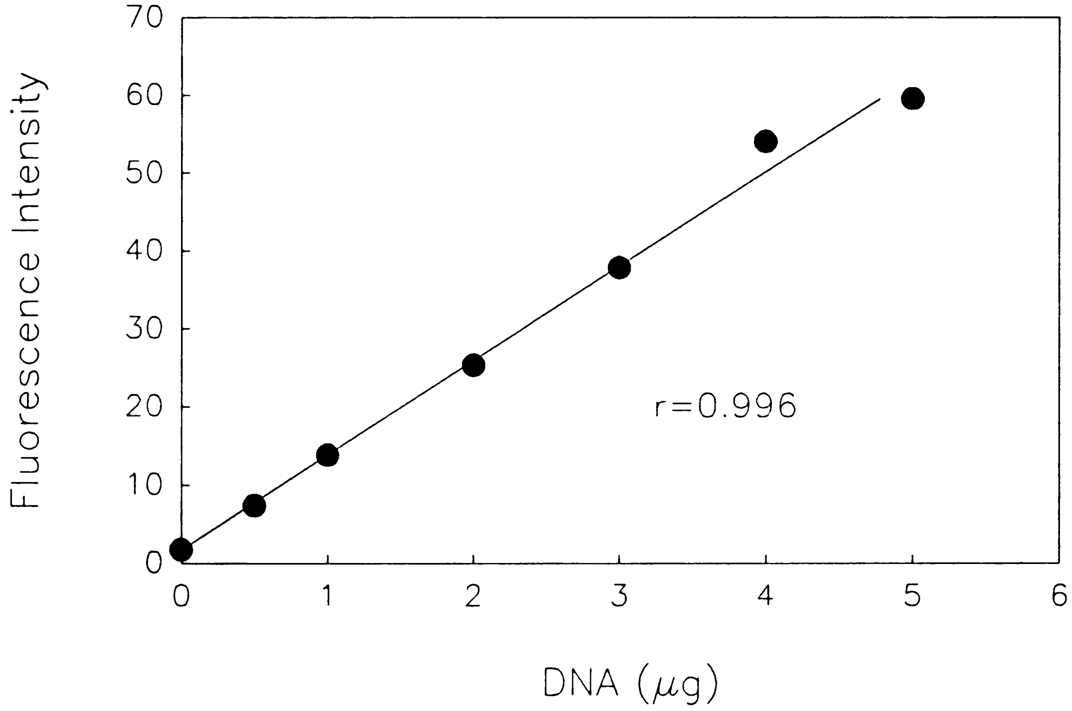
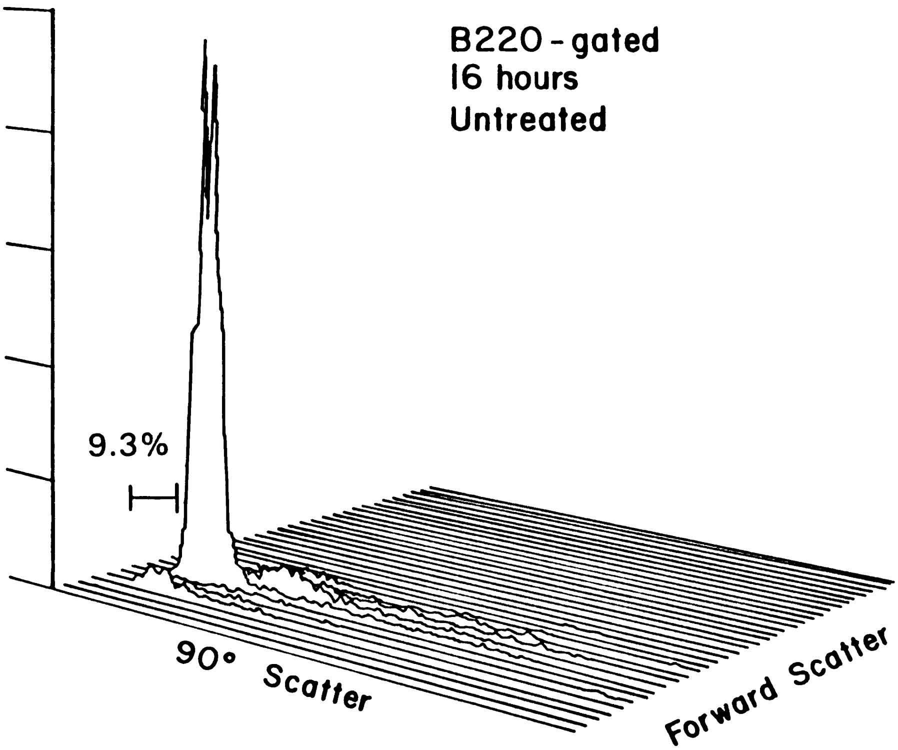

70
60
50
40
30
20
10
0
Fluorescence Intensity
r=0.996
0
1
2
3
4
5
6
DNA (μg)

Figure 2.2 Flow cytometric light-scatter profiles of B220$^+$ cells 16 hours after culture with or without 0.1 μM dexamethasone. Bone marrow cells were phenotypically labelled with B220 antibody coupled to FITC, fixed in ethanol, and stained with propidium iodide. Scatter profiles are only for gated cells which bound the B220 antibody. Forward scatter is an indication of cell size while 90° scatter indicates the granular content of the cells. The proportion of "small" B220$^+$ cells is indicated underneath the bars. Data are representative of 6 separate experiments.


B220-gated
16 hours
Untreated
Cell Count
9.3%
90° Scatter
Forward Scatter

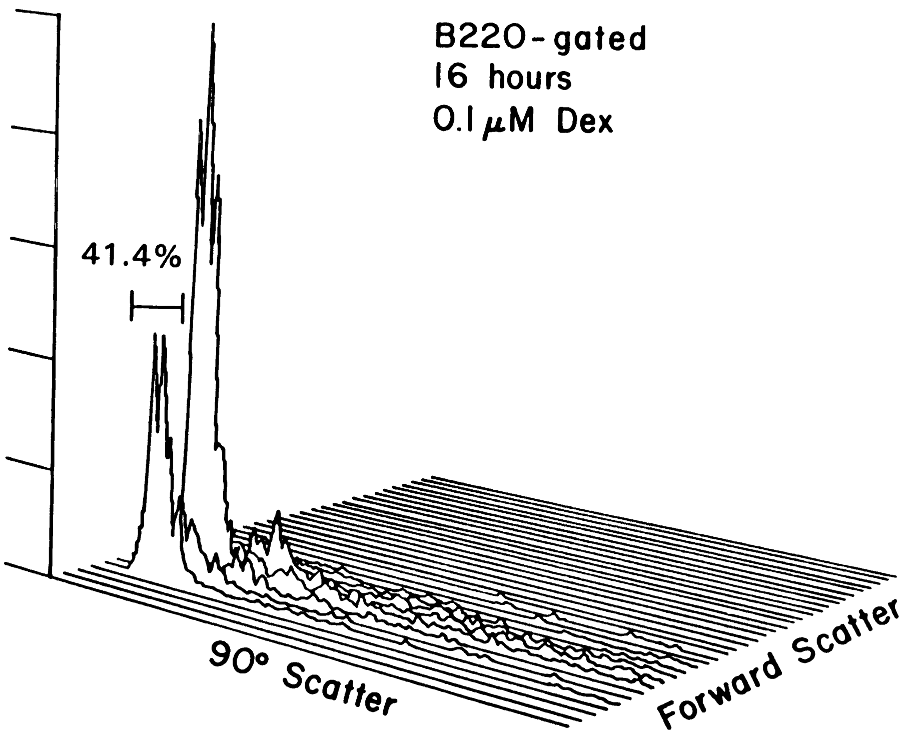
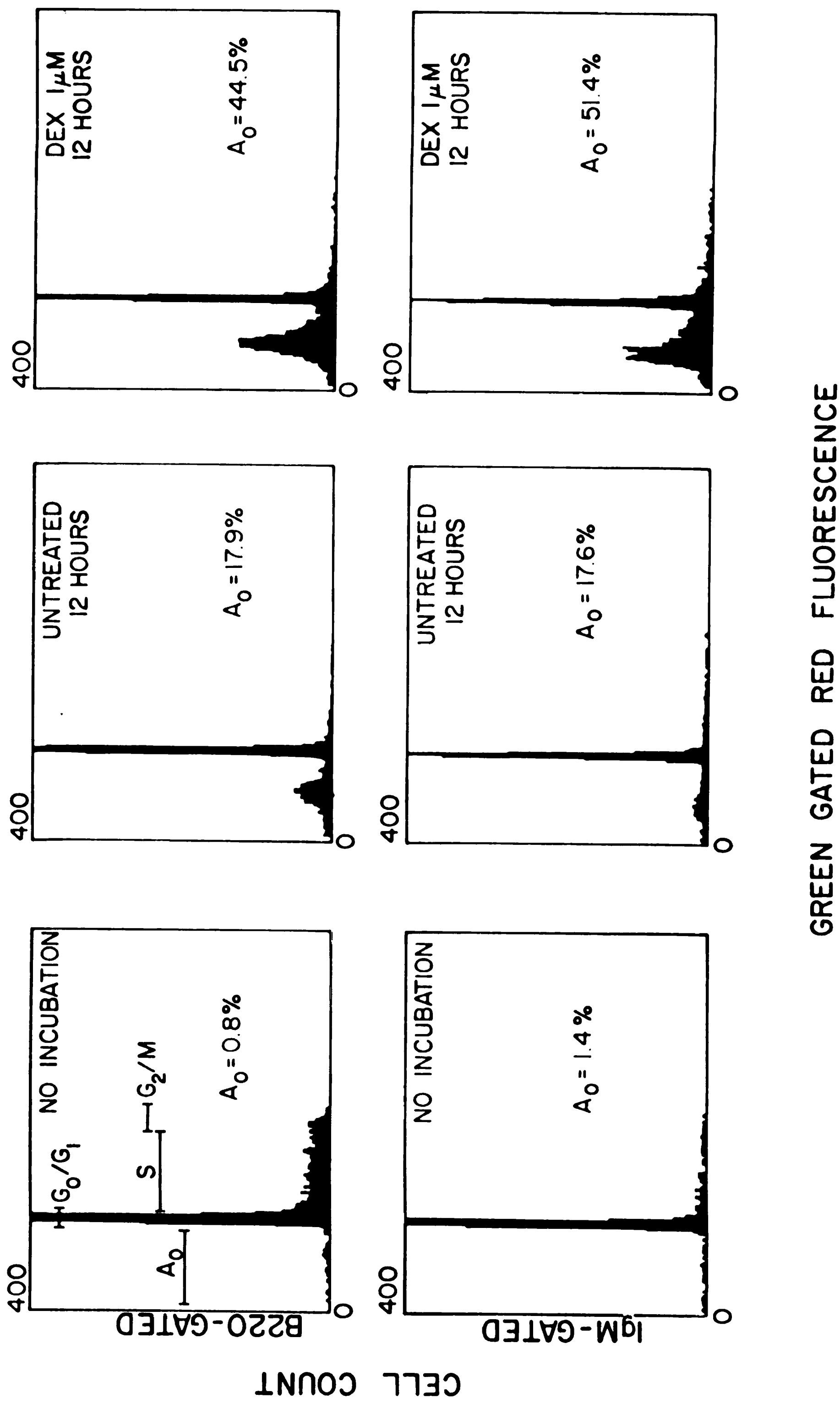

B220-gated
16 hours
0.1 μM Dex
Cell Count
41.4%
90° Scatter
Forward Scatter

Figure 2.3 Cell cycle histograms of B220- and IgM-gated bone marrow cells. After 12 hours of culture with or without 1 μM Dx, bone marrow cells were labeled with anti-IgM or B220 antibodies coupled to FITC (green fluorescence), fixed in ethanol, stained with propidium iodide (red fluorescence), and analyzed using flow cytometry. Histograms represent cell cycle analysis of either B220$^+$ (top row) or IgM$^+$ (bottom row) lymphocytes gated to exclude nonspecific antibody binding based on forward and 90° scatter of fluorescence positive cells (as in Figure 1). Histograms are based on 5000 or 2000 total B220$^+$ or IgM$^+$ events, respectively. Phases of the cell cycle are indicated in the B220 gated, no-incubation histogram for freshly prepared bone marrow. For each histogram the proportion of fluorescent cells in the A_0 region is indicated. Data are representative of 4 separate experiments.



NO INCUBATION
G_0/G_1
S
G_2/M
A_0
A_0=0.8%
UNTREATED 12 HOURS
A_0=17.9%
DEX 1 μM 12 HOURS
A_0=44.5%
NO INCUBATION
A_0=1.4%
UNTREATED 12 HOURS
A_0=17.6%
DEX 1 μM 12 HOURS
A_0=51.4%
400
0
B220-GATED
IgM-GATED
CELL COUNT
GREEN GATED RED FLUORESCENCE

Figure 2.4 Kinetic response of B220- and IgM-gated bone marrow cells to induction of apoptosis by 1 μM dexamethasone during first 10 hours of exposure. Cells were phenotypically labelled with antibodies to B220 or IgM, fixed, and stained with propidium iodide. Flow cytometry was used to quantitate the proportion of phenotype-positive lymphocytes in the A_0 region of the cell cycle. Data are representative of two separate experiments.

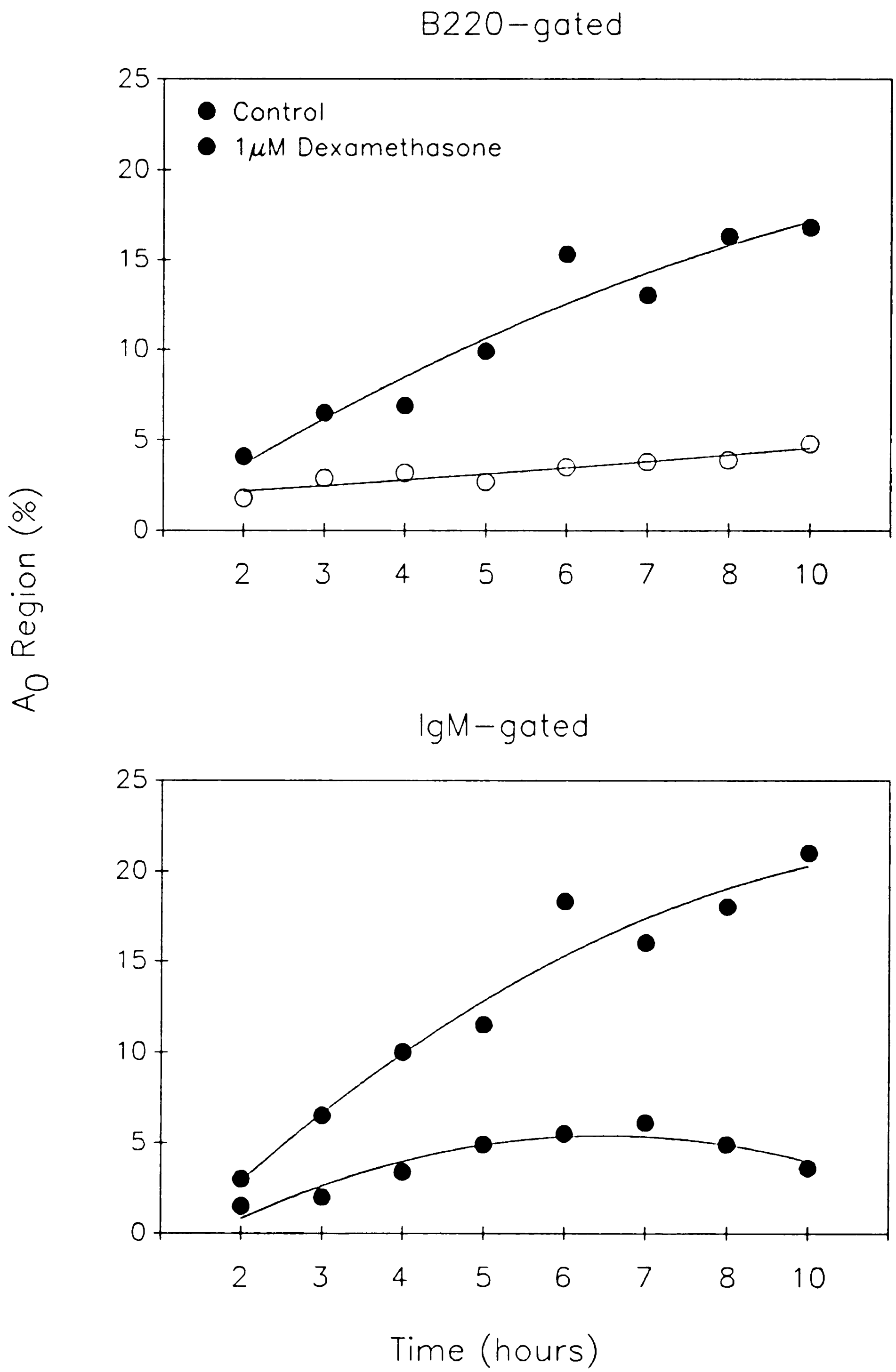

B220-gated
Control
1μM Dexamethasone
25
20
15
10
5
0
2
3
4
5
6
7
8
10
IgM-gated
25
20
15
10
5
0
2
3
4
5
6
7
8
10
A0 Region (%)
Time (hours)

Figure 2.5 Kinetic response of B220-, IgM-, and IgD-gated bone marrow cells 12 to 48 hours after treatment with or without 1 μM dexamethasone. Cells were phenotypically labeled with antibodies to B220, IgM or IgD. After fixation, cells were stained with propidium iodide and analyzed using flow cytometry to quantify the proportion of phenotype-positive cells in the A_0 region of the cell cycle.

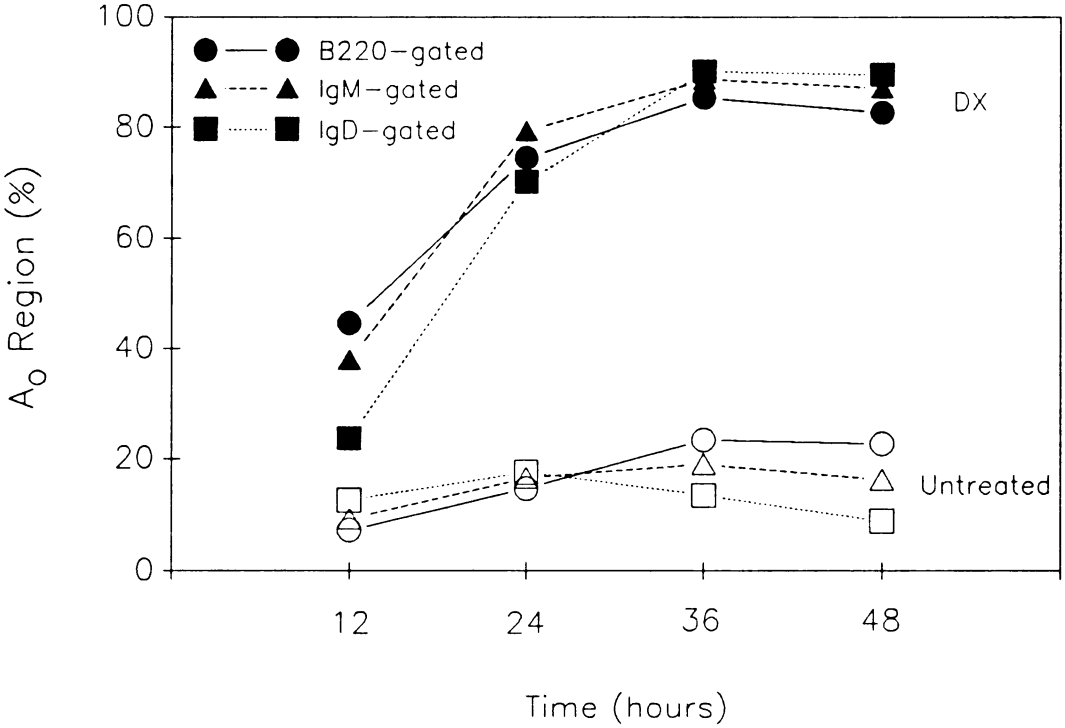
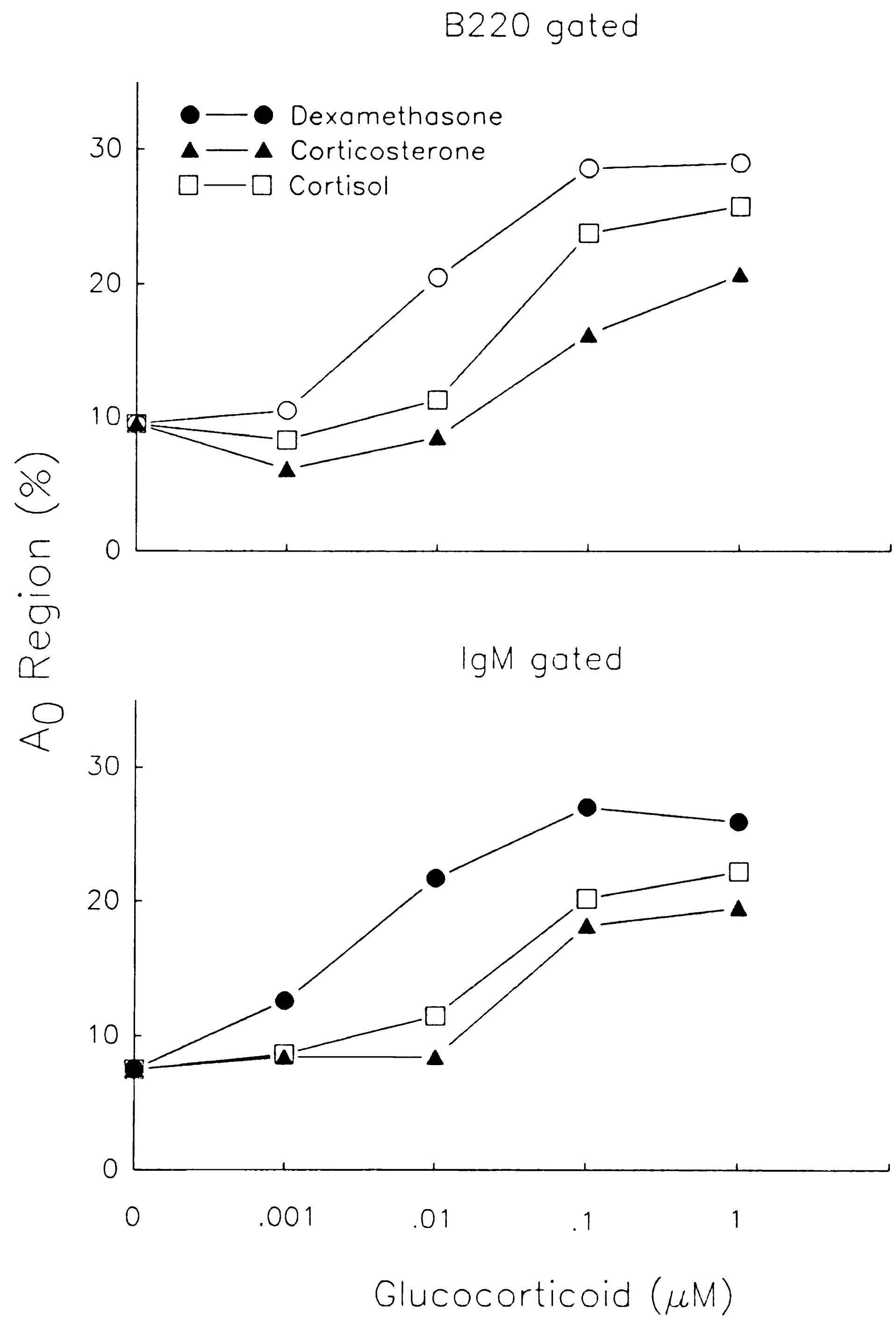

100
80
60
40
20
0
A_o Region (%)
B220−gated
IgM−gated
IgD−gated
DX
Untreated
12
24
36
48
Time (hours)

Figure 2.6 Dose response of B220- and IgM-gated bone marrow cells incubated for 8 hours with the indicated concentrations of dexamethasone, corticosterone, or cortisol. Cells were phenotypically labeled with antibodies to B220 or IgM, fixed, and stained with propidium iodide for cell cycle analysis. Flow cytometry was used to determine the proportion of phenotye-positive lymphocytes in the A_0 region of the cell cycle. Data are representative of 2 separate experiments.



B220 gated
Dexamethasone
Corticosterone
Cortisol
IgM gated
A0 Region (%)
0
10
20
30
0
.001
.01
.1
1
Glucocorticoid (μM)

Figure 2.7 Response of B220- and IgM-gated bone marrow cells to induction of apoptosis when treated with with or without 0.1 μM dexamethasone for up to 16 hours. The ability of the glucocorticoid receptor antagonist RU 38486 (1 μM) to block apoptosis also was tested. Cells were labelled phenotypically with antibodies to IgM or B220, fixed, and stained with propidium iodide. Flow cytometry was used to quantify the percentage of phenotype positive lymphocytes in the A_0 region of the cell cycle. Data are representative of 4 separate experiments.

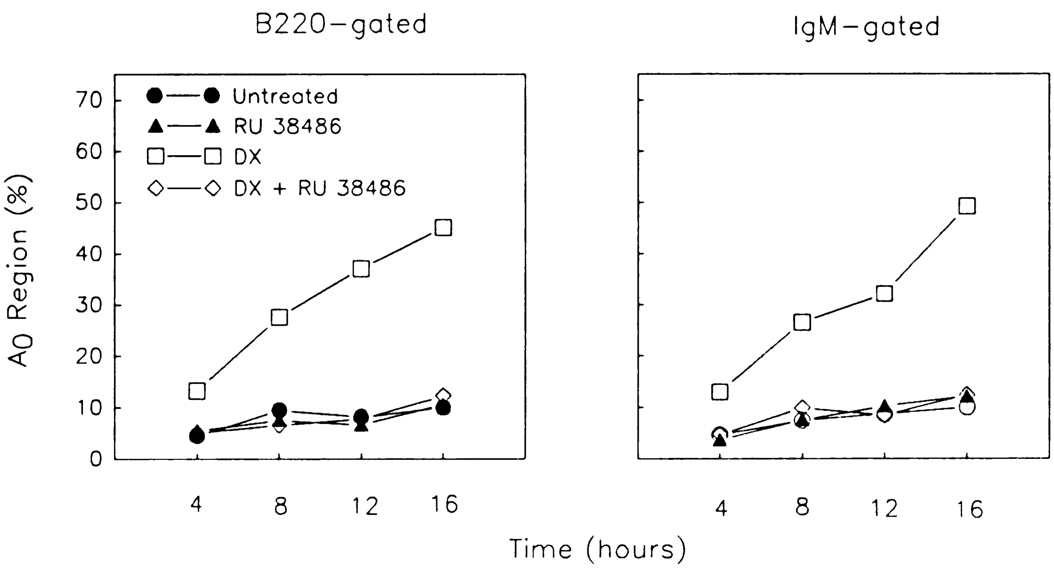
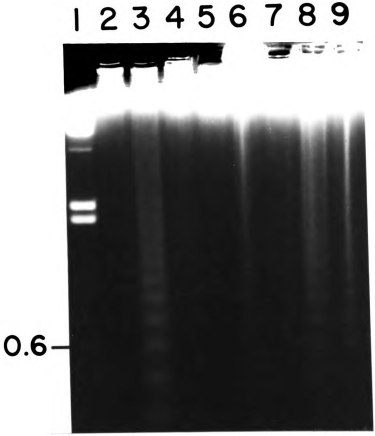

B220−gated
IgM−gated
Untreated
RU 38486
DX
DX + RU 38486
A0 Region (%)
70
60
50
40
30
20
10
0
4
8
12
16
Time (hours)

Figure 2.8 Detection of 200 base pair fragmentation of DNA of lysates of B220$^+$ cells which had been treated with dexamethasone. Bone marrow cells were incubated for 5 hours with 1 μM Dx. Subsequently, B220$^+$ cells were fractionated from whole bone marrow by panning. Cells were lysed and DNA extracted and purified prior to electrophoresis on agarose gels. Lane 1, molecular weight markers from a HINDIII restiction digest of λ phage DNA, a 0.6 kb fragment of DNA is noted; lane 2, untreated thymocytes; lane 3, dexamethasone-treated thymocytes; lane 4, untreated whole bone marrow; lane 5, untreated B220$^+$ cells; lane 6, untreated B220$^-$ bone marrow; lane 7, dexamethasone-treated whole bone marrow; lane 8, dexamethasone-treated B220$^+$ cells; lane 9, dexamethasone-treated B220$^-$ bone marrow cells. Data are representative of 2 separate experiments.


1 2 3 4 5 6 7 8 9
0.6

Chapter 3

Suppression of Murine B-cell Lymphopoiesis by Chronic Elevation of Plasma Corticosterone

SUMMARY

Pellet implants in mice were used to mimic plasma corticosterone (CS) levels found during chronic stress (30-100 μg/dl). Severe thymic atrophy was apparent within 24 hours of implantation, and by day 3 thymus weights were less than 20% of sham controls. Phenotypic analysis of bone marrow (BM) B-lineage lymphocytes by flow cytometry indicated a 40% decrease in BM Ig^+ cells and a 70% decrease in $B220^+$ cells by day 3 which was accompanied by a two-fold increase in the mean fluorescent intensity for both markers. A residual population of Ig^+ cells which remained in the BM of CS-treated mice from day 5 to 15 were examined using two-color flow cytometric analysis. The $B220^+Ig^-$ precursor B-cells had been completely depleted by day 5 and the remaining cells were $B220^{bright}IgM^+IgD^{bright}$. To determine if this depletion was due to disruption in cell cycling and/or apoptosis, phenotype-gated flow cytometric cell cycle analysis was performed at earlier time points. Between 6 and 36 hours after CS implantation the appearance of a small but distinct population of $B220^+$ and IgM^+ cells in the "hypodiploid" region of the cell cycle was noted which was previously termed the A_0 region and corresponded to cells undergoing apoptosis. The proportion of $B220^+$ cells in the S phase of the cell cycle also declined sharply during this period. These data indicate that chronic elevation of plasma CS caused depression of B-lineage lymphopoiesis by depletion of cycling, precursor and immature

B-cells in murine BM at least in part by induction of apoptosis.

INTRODUCTION

Chronic stress caused by malnutrition, burns, trauma, surgery, etc. is characterized by elevated plasma glucocorticoid (GC) levels and suppressed immune function associated with lymphopenia (DePasquale-Jardieu & Fraker, 1980; Kagan *et al.*, 1989; Alleyne & Young, 1967). The role GC may play in regulating immune function, especially lymphopoiesis, has been of great interest recently since these steroids readily induce programmed cell death (or apoptosis) in thymocytes (Wyllie, 1980; Compton & Cidlowski, 1986) and in B-lineage cells *in vitro*, as reported in the previous chapter of this dissertation. While it is well documented that GC cause atrophy of the more immature cells of the thymic cortex (Weissman, 1973) much less is known about the *in vivo* effects of GC on the developing B-lineage cells of the BM marrow.

A single injection of 15 mg of cortisol (HC) into mice has been reported to cause a decrease in the proportion of bone marrow cells expressing cytoplasmic μ but not sIgM (Ku & Owen, 1986), while dexamethasone (DX) administered as one dose or as multiple daily injections caused a significant decrease in percentage of BM surface IgM-bearing cells (Sabbele *et al.*, 1987). Vines *et al.* (1980) reported that a single injection of 1 mg DX into rats resulted in depletion of BM TdT^+ cells. These studies suggested that pharmacological levels of GC *in vivo* resulted in depletion of three different subpopulations of developing B-lineage cells but offered little information regarding the stage (or stages) of development affected. Also, the mechanism responsible for this depletion was not defined.

While such studies have provided some insight into the role GC play during stress, there was little information regarding the actual *in vivo* effects of chronic exposure of natural GC within physiological ranges. Further, it was unknown if the precursor B-lineage cells might be more sensitive to GC than are the more mature B-cells, as is the case for thymocytes. The present studies utilized a pellet implantation system that delivered levels of CS (the natural GC predominant in mice) (Spackman & Riley, 1978) that were analogous to those observed during chronic stress (DePasquale-Jardieu & Fraker, 1980; Kagan *et al.*, 1989). This system allowed for the flow cytometric analysis of changes in subpopulations of B-lineage cells during conditions mimicking chronic stress. Further, since BM B-lineage cells were shown in Chapter 2 to be induced to undergo apoptosis by GC *in vitro*, the same method for examining the cell cycle was used to determine if B-lineage cells undergo apoptosis as a result of *in vivo* administration of GC and if indeed the rate of cycling of large pre-B cells also is altered. And finally, the ability of BM B-cells to respond to antigenic stimulation was examined to determine the effects of GC on the functional capacity of residual cells.

METHODS

Corticosterone Pellet Implantations

A/J or CAF_1 male mice (The Jackson Laboratories, Bar Harbor, ME) 5-7 weeks of age were placed under methoxyflurane anaesthesia (Pittman-Moore, Mundelein, IL) and subcutaneously implanted with pellets made of 20 mg CS (Sigma, St. Louis, MO) compressed into a 20 mg cholesterol matrix (Sigma). Sham control mice were implanted with pellets containing only cholesterol. Upon recovery, mice were housed in sterilized cages and maintained on acidified water and commercial rodent chow (Purina, St. Louis, MO) in a quiet, temperature-controlled room.

Plasma Corticosterone Assay

In order to minimize the release of endogenously produced CS during handling of the mice, blood was collected into heparinized tubes by severing the subclavian vein of mice under anaesthesia within 90 seconds of contacting the cage. Blood samples were collected from the sham control and CS-implanted mice between 8-9 A.M. and the resulting plasma was stored at -20° until analysis. In some experiments white blood cell (WBC) counts were determined using 25 μl aliquots of whole blood via the Unipette system as directed by the manufacturer (Becton Dickinson,) which employs acetic acid to lyse red blood cells after which WBC are determined microscopically under phase contrast.

Using a modification of the method of DePasquale-Jardieu & Fraker (1980), CS was extracted from 30 μl plasma by vigorous shaking with 600 μl dichloromethane (Aldrich, Milwaukee, WI). After centrifugation at 500 xg the aqueous phase was discarded and the organic phase treated with 100 μl 0.1N NaOH. The organic phase was separated by centrifugation and fluorescence was developed by vigorous mixing with 200 μl of a mixture containing 3 parts concentrated H_2SO_4 and 1 part absolute ethanol (Aldrich, Milwaukee, WI). Thirty minutes later the acid phase was collected and fluorescence intensity was measured on a Perkin-Elmer 650-40 Spectrofluorimeter using an excitation wavelength of 475 nm and emission of 525 nm. A standard curve of 15-100 ng CS was used to calculate plasma concentrations which gave a linear response within the range of CS found in the plasma samples (Figure 3.1). An internal standard of 25 ng CS included in each assay indicated that yields ranged from 70% to 100%.

Immunphenotypic Labelling of Bone Marrow B-lineage Cells

Bone marrow was flushed from femurs and washed with phosphate buffered saline, pH 7.4, containing 2% fetal calf serum. Contaminating red blood cells were removed by density gradient centrifugation over Histopaque 1083 (Sigma, St. Louis, MO) or lysed (for cell cycle determinations) using 0.16 M NH_4Cl containing 0.17 M Tris, pH 7.2. After washing, aliquots of 10^6 cells in PBS containing 5% heat-inactivated FCS and 0.15% NaN_3 were incubated with 150 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM+G (2 μg, Tago, Burlingame, CA), biotin-conjugated goat anti-mouse IgM (0.9 μg, Cappel, Malvern, PA), biotin conjugated rat anti-mouse B220 (1.5 μg, purified from supernatants of the RA3.6B2 cell line, a gift from R.A.Miller, Ann Arbor, MI), or FITC conjugated sheep anti-mouse IgD absorbed against mouse IgM (2 μg, The Binding Site, Birmingham, England) for 30 minutes at 4°C. The cells were washed twice by centrifugation at 400 xg for 5 minutes, resuspended in 1 ml label buffer and held on ice until analysis. When biotinylated antibodies were used, the cells were incubated with streptavidin-conjugated FITC (Av-FITC, Vector, Burlingame, CA) or phycoerythrin (Av-PE, Vector) for 30 minutes at 4°C.

Preparation of B220$^+$ and sIgM$^+$ Bone Marrow B-cells for Cell Cycle Analysis

Phenotype-gated cell cycle analysis was performed on bone marrow as described in Chapter two. The cells were labelled with biotinylated anti-IgM or anti-B220 and Av-FITC as above, fixed in 50% ethanol, and stored at 4°C until analysis. On the day of analysis, which was performed within 48 hours of fixation, the cells were washed, DNA stained in 1 ml of a propidium iodide (PI) staining reagent (PBS pH 7.4 containing 0.05 mg/ml RNase A at 50 units/mg and 50 μg/ml PI) at room temperature, and stored on ice until analysis.

Flow Cytometric Analysis of Bone Marrow B-lineage Cell Phenotype and Cell Cycle Status

One- and two-color fluorescent samples were analyzed on a linear scale using an Ortho Cytofluorograph 50H with a 2150 computer system or on a logarithmic scale using an 80386 computer system and Acqcyte software (Phoenix Flow Systems, San Diego, CA), respectively. Cell size and granularity were assessed by low-forward scatter of a helium-neon or argon laser and low-side scatter of an argon laser. FITC and PE were excited with the 488 nm line of an argon laser operating at 300 mWatt regulated output and emission detected at 530±15 nm and 615 nm, respectively. Background fluorescence was determined using Av-FITC- or Av-PE-labelled bone marrow samples. Thymocytes were used as controls for gating purposes. All analyses were based on at least 10,000 events.

Cell cycle analysis for apoptotic cells was performed as described in Chapter two. Phenotype-positive bone marrow cells were gated using two-color cytograms and light-scatter profiles and were analyzed for PI fluorescence at 488 nm excitation with emission detected at 620-700 nm. Analyses were based on 2000-5000 phenotype-positive lymphocytes.

Analysis of Functional Capacity of Bone Marrow B-cells

In some studies the antigenic response of BM B-cells in sham control and CS-treated mice was assessed *in vitro* using a modification of short term bone marrow culture system described by Medina *et al.* (1988). BM was flushed from the femurs and tibias as described, red blood cells removed, and cells pipetted into 24 well culture plates in a RPMI-1640-based medium (for greater detail see Chapter 4). The cells were stimulated with 0.01 μg/ml trinitrophenol-lipopolysaccharide (TNP-LPS) and were cultured under an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 at 37°C. Five days later the cells were removed from culture by gentle aspiration from the wells, pelleted by centrifugation at 400 xg for 5 minutes, and resuspended in 75 μl Hanks balanced salt solution, pH 7.2, supplemented with 1% heat-inactivated FCS.

In other studies mice were injected intraperitoneally with 5 μg TNP-LPS 6 days after pellet implantation. At day 10 BM was flushed from femurs and tibias with PBS plus 2% HIFCS. BM cells were washed by centrifugation at 400 xg for 5 minutes and resuspended in Hanks buffered saline, pH 7.2, supplemented with 1% heat inactivated FCS.

Response to TNP-LPS was determined using a plaque forming cell assay decribed in detail in Chapter 4. Briefly, aliquots of BM cells were incubated on agarose plates with TNP coupled to sheep red blood cells at 37°C for 2 hours followed by incubation with guinea pig complement for 1 hour. Cells producing antibody to TNP were enumerated by counting the number of plaques formed on each plate. Data are expressed as number of anti-TNP plaque-forming cells (PFC) per 10^7 cells or per organ as appropriate.

Statistical Analysis

Student's *t-tests* were used to determine significant differences ($p<0.05$) between sham-control and CS-treated mice.

RESULTS

Plasma Corticosterone Concentrations

Plasma corticosterone concentrations in CS-implanted mice rose to 142 μg/dl six hours post-implantation, declined to approximately 70 μg/dl at 24 hours, reached a plateau of around 30 μg/dl days 3-10, and by day 15 were near control levels (Table 3.1). Thus, plasma CS remained chronically elevated for almost two weeks and within a range consistent with physiological levels seen in burn patients, malnourished children, and zinc deficient mice (Alleyne & Young, 1967; Kagan *et al.*, 1989; DePasquale-Jardieu & Fraker, 1980). In the CS-implanted mice thymic atrophy was evident 12 hours after implantation and declined to less than 15% of the thymus weight of controls by day 10 (Table 3.1). A significant proportion of thymocytes underwent apoptosis within 24 hours of CS pellet implantation (not shown). This finding is consistent with previous observations which indicated that elevated plasma glucocorticoids caused thymic atrophy by inducing apoptosis (Compton & Cidlowski, 1986). Additionally, spleen weight and white blood cell counts were reduced in CS-treated mice by about 50%, when assessed at days 5 and 10 after pellet implantation, indicating that significant lymphopenia had occurred in the peripheral lymphoid tissues (Table 3.2).

Flow Cytometric Analysis of Bone Marrow B-lineage Subpopulations

BM from CS treated mice at day 5 underwent a significant population shift when analyzed for cell size (forward scatter) and granular contents or density (side scatter) (Figure 3.2). The proportion of small, non-granular cells (lymphocytes) declined from 33% in controls to 18% of total BM cells in CS-treated mice and was accompanied by an increase in the proportion of large, granular cells (Figure 3.2) suggesting a significant shift in the BM subpopulations and resulting in the loss of lymphocytes.

Analysis of subpopulations of BM B-lineage cells from CS-treated mice using fluorescent antibodies indicated a 40% decrease in surface immunoglobulin (Ig)-bearing cells and a 70% loss in $B220^+$ cells by day 3 post-implantation (Figure 3.3). The decrease in proportion of $B220^+$ and Ig^+ cells represented a loss in the absolute number of B-lineage cells since the BM cellularity was unchanged by CS treatment (Table 3.2). There was no additional depletion of Ig^+ or $B220^+$ cells in the bone marrow of CS-treated mice from days 3-15 (Figure 3.3), though plasma CS levels remained elevated through day 10 before decreasing by day 15 (Table 3.1). This suggests the presence of a residual population of B-lineage cells which was CS resistant. Surprisingly, the Ig and B220 fluorescent intensity of the residual cells, which represented less than 5% of total BM cells, was twice that of control B-cells from days 3-15 and suggested a greater mean density of these molecules on the cell surface (Figure 3.3).

In order to determine more accurately the phenotype of the residual population of B-cells, BM cells at day 5 post implantation were fluorescently labelled with anti-IgM, anti-IgD or anti-B220 antibodies. Two-color cytograms indicated that a residual population of cells from CS-treated mice, which represented 3.9% of total BM cells were B220$^+$ IgM$^+$, and that the B220$^+$ IgM$^-$ population was depleted (Figure 3.4). The residual B220$^+$ IgM$^+$ cells were also IgD$^+$ while IgM$^+$ IgD$^-$ immature B-cells were depleted (Figure 3.4). Interestingly, the proportion (and absolute number) of IgD$^+$ cells in the bone marrow increased about two-fold (Figure 3.4). It was found that B220$^+$ cells with a low fluorescent intensity were preferentially depleted so that the residual cells had a relatively high mean fluorescent intensity for B220 (B220^{bright}) (Figure 3.4) thus verifying results previously obtained (Figure 3.3). The mean fluorescent intensity of bone marrow IgD$^+$ cells was significantly higher (IgD^{bright}) in CS-treated mice than in controls, and low intensity IgD$^+$ cells also had been depleted in th experimental mice (Figure 3.4). The fluorescence intensity of the residual IgD^{bright} cells was similar to that found in splenocytes of control mice (data not shown). The fluorescence intensities of IgM$^+$ cells in the BM of CS-treated mice, however, were distributed throughout a range comparable to that of IgM$^+$ cells from BM of control mice

(Figure 3.4). Taken together, these data indicate that the residual B-lineage cells in the BM of CS-treated mice at day 5 were B220^{bright}IgM^{+}IgD^{bright} and that the proportion of these cells was at least two-fold higher than in the bone marrow of sham controls.

Detection of Apoptosis Using Two-Color Cell Cycle Analysis

GC are known to cause cell cycle arrest in lymphoid cell lines (Harmon *et al.*, 1979) and were shown in the previous chapter of this dissertation to cause apoptosis in cultured BM B-lineage cells. To determine if the depletion of pre-B and immature B-cells observed *in vivo* was due to alterations in the cell cycle and/or apoptosis, phenotype-gated cell cycle analysis was performed on BM from mice at 6, 12, 18, 24, and 36 hours after pellet implantation. Early time points were chosen in an attempt to examine the BM B-lineage cells as they disappeared, a process that was found to be completed by day 3 (Figure 3.3). B220- and IgM-gated bone marrow cells from CS-treated mice formed a small but distinct population of cells in the "hypodiploid" region of the cell cycle when analyzed using PI fluorescence histograms (Figure 3.5). The region to the left of G_0/G_1 has been previously termed the A_0 region of the cell cycle and has been shown to correspond to the appearance of apoptotic cells in the thymus (Telford *et al.*, 1991). The proportion of both IgM^{+} and B220^{+} cells in the A_0 region 12 hours after pellet implantation was around

4%, plateaued through 24 hours, and then declined to near control levels (Figure 3.6). Though the proportion of B-lineage cells in the A_0 region appeared to be small, it represented a 2-5 fold increase over sham controls which had less than 1% of B220$^+$ and 1.5% of IgM$^+$ BM cells in the A_0 region through 36 hours (Figure 3.6). The highest levels of B220$^+$ cells in the A_0 region, between 6-24 hours after pellet implantation, corresponded to a 50% decrease in percentage of B220$^+$ cells in the BM of CS-treated mice (Figure 3.6). Interestingly, the percentage of IgM$^+$ cells in the BM decreased between 6 and 12 hours, increased slightly at 18 hours, and then did not change through 36 hours (Figure 3.6). This small change in the proportion of IgM$^+$ cells coincided with a significant increase in the proportion of cells in the A_0 region of the cell cycle. Subsequent analysis at 24 hours indicated that while the proportion of IgM$^+$IgD$^-$ immature B-cells had decreased, they were replaced by IgM$^+$IgD$^+$ B-cells (data not shown) giving the appearance that there were no changes in the proportion of IgM$^+$ cells. These data indicated that pre-B and immature B-cells were induced to undergo apoptosis by CS but that IgM$^+$IgD$^+$ cells were resistant.

Finally, there was a decrease in the proportion of precursor B-cells in CS-treated mice which were actively cycling. By 24 hours after pellet implantation less than 4% of B220$^+$ cells were in S phase compared to over 13% in S phase for control BM (Table 3.3, see also Figure 3.5). At day

5 the proportion of $B220^+$ cells in S phase dropped to less than 1%. It is unclear whether this decrease was due to S phase cells entering apoptosis or the arrest of cycling cells in the G_0/G_1 phase. Regardless, these data demonstrate that CS at chronic physiological levels caused a significant loss in cycling pre-B-cells.

Functional Capacity of Residual Bone Marrow B-cells

The ability of residual BM cells to respond to the T-cell-independent antigen, TNP-LPS, was evaluated using a short term culture system. The numbers of anti-TNP PFC were slightly higher per 10^7 cells in the BM of CS-treated mice compared to controls (Table 3.4). These data suggested that there was an increase in the number of antigen-responsive cells present in the BM of CS-implanted mice. This was particularly interesting since the proportion of B-lineage cells was significantly reduced in the CS-treated mice though the number of mature $sIgD^+$ cells doubled (Figure 3.4). Since it is recognized that the culture system used in these studies removed the cells from their CS-rich microenvironment, cells were challenged *in vivo* by an injection of TNP-LPS at day 6 after pellet implant and a PFC assay was performed at day 10. Anti-TNP PFC production in the BM of CS-treated mice was nearly two-fold higher than in control BM (Table 3.4). This corresponded to the two-fold increase in IgM^+IgD^+ BM B-cells and indicates an increase in the number of antigenic responsive cells in the BM of CS-implanted mice.

DISCUSSION

B-cell lymphopoiesis proceeds through a number of phenotypically distinct stages (Kincade *et al.*, 1989). The cues that control development of actively cycling, B220^{+} Ig^{-} pre-B-cells to noncycling, surface Ig^{+} virgin B-cells are poorly understood but appear to be regulated by a combination of cytokines together with immunoglobulin gene rearrangement (Era *et al.*, 1991; Rolink *et al.*, 1991). Chronic stress appeared to alter this developmental pathway since malnutrition and physical trauma caused lymphopenia (DePasquale-Jardieu & Fraker, 1980; Kagan *et al.*, 1989). The increased levels of circulating GC that appear with stress are likely candidates for alterating B-cell development since they have been shown to have detrimental effects on developing T-cells (Weissman, 1973; DePasquale-Jardieu & Fraker, 1980; Compton & Cidlowski, 1986). However, previous studies used acute or pharmacological doses of GC while the pellet implantation system employed here released CS, the natural GC found in mice (Spackman & Riley, 1978), at physiological levels for at least 10 days. Even the initial spike of CS (142 μg/dl) was not that different from plasma concentrations previously reported to be over 100 μg/dl in zinc deficient mice (DePasquale-Jardieu & Fraker, 1980). CS levels declined to two to three times control values by 24 hours and remained constant through 10 days.

The data show that persistent, moderate elevation in plasma CS concentrations consistent with physiological stress levels severely depleted the bone marrow B-lymphocyte compartment. There was a significant shift in the the proportion of small lymphocytes in the BM to larger, more granular cells. $B220^{+}Ig^{-}$ pre-B cells were completely lost from the BM of CS-treated mice as were immature $B220^{+}IgM^{+}IgD^{-}$ B-cells representing a significant alteration in B-cell lymphopoiesis. Although precursor B-lineage cells were depleted as early as day 3 after pellet implantation, a residual population of Ig^{+} cells remained past day 15. The nature of this residual population is unknown; however, it is unlikely that these cells were newly developed since cycling pre-B cells disappeared as early as 24 hours after pellet implantation (Table 3.3). This represented a 10-fold drop in the absolute number of $B220^{+}$ S phase cells per femur at 24 hours after CS implantation and a nearly 30-fold decrease at day 5. This would nearly eliminate the turnover of pre-B cells in the BM which is normally around 6%/hour (Opstelten & Osmond, 1983).

Alternatively, these residual $B220^{bright}IgM^{+}IgD^{bright}$ cells may have come from the periphery. A population of long-lived $IgM^{+}IgD^{bright}$ B-cells has recently been described which may represent as much as two-thirds of the peripheral B-cell pool and is thought to be a form of memory cell derived from a T-independent selection process (Gu *et al.*, 1991; Forster & Rajewsky, 1990). Conversely, these cells may represent a more mature population of resident BM cells that is resistant to CS. Nevertheless, the presence of this seemingly more mature population of B-cells in the CS-treated BM may account for its unimpaired response to the T-independent antigen TNP-LPS.

Phenotype-gated flow cytometric cell cycle analysis made it possible to detect the presence of apoptotic cells in BM B-cells. This technique has proven extremely useful for detecting low levels of apoptosis in heterogeneous populations of cells (Telford *et al.*, 1991). The data reported here indicated that a small, but significant proportion of $B220^+$ cells underwent apoptosis which was ongoing between 6 and 36 hours after CS pellet implantation. The increase in the percentage of cells in the A_0 region of the cell cycle coincided with a drop in the proportion of B220-bearing BM cells. Both $B220^+$ and IgM^+ cells were about 10% of the total BM population at 36 hours suggesting that pre-B cells had been depleted by that time and the remaining cells were $B220^+IgM^+$. Interestingly, the appearance of IgM^+ cells in the A_0 was similar in kinetics to that of the $B220^+$ cells, but the proportion of IgM^+ cells in the BM dropped slightly and then remained constant through 36 hours. This may have been due to slower kinetics of clearance of apoptotic IgM^+ cells, movement of cycling precusors toward maturity, or recirculation of Ig bearing cells into the BM. Subsequent analysis at 24 hours indicated that immature IgM^+IgD^-cells had been replaced by IgM^+IgD^+ cells; however, the origin of these mature B-cells was not immediately obvious. Contrary to these results, it was found in the previous chapter of this dissertation that IgD^+ cells underwent apoptosis when exposed to GC *in vitro*. However, these cells may have represented newly formed B-cells while the residual cells in the bone marrow from CS-implanted mice were possibly long-lived cells. Regardless, it is apparent that tissue culture provides a much different environment whic is not always a reflection of the *in vivo* situation.

The proportion of B-lineage cells from CS-treated mice appearing in the A_0 region of the cell cycle was considerably lower than seen in cultured thymocytes (Telford *et al.*, 1991) and BM B-lineage cells as reported in the previous chapter of this dissertation. This is not surprising since there have been reports that apoptotic cells are rapidly cleared by phagocytic cells (Duvall *et al.*, 1985; Savill *et al.*, 1989). The intact microenvironment of the BM is rich in phagocytic cells and may be uniquely suited for clearance of these cells. The disappearance of apoptotic cells was fairly rapid here since $B220^+$ cells were depleted to 50% of control values over a 24-hour period, while only slightly over 4% of the $B220^+$ cells were found in the A_0 region at any one time. The viability of whole BM determined by trypan blue exclusion over the 24-hour time period was always greater than 90% suggesting that apoptotic cells were cleared from the BM microenvironment before deterioration.

It is clear that lymphopoiesis was significantly altered since cycling pre-B and immature B-cells were selectively depleted from the BM of mice chronically exposed to CS; however, it is yet not clear how far back along the developmental pathway these effects extended. There is some evidence that GC will deplete rat BM of terminal deoxynucleotidyl transferase positive-cells but whether these very early lymphocyte precursors undergo apoptosis is unknown (Vines *et al.*, 1980). While it appears that GC represent important regulators of B-cell lymphopoiesis, it is unclear as to why precursor lymphocytes were susceptible to GC-induced apoptosis while more mature cells appeared to be resistant. There is evidence that precursor and immature B-lineage cells may utilize one or more "apoptotic pathways" since apoptosis can also be induced by anti-IgM in WEHI 231 cells (an immature B-cell line), growth factor deprivation in immortalized pre-B-cells, and possibly by nonproductive gene rearrangements (Benhamou *et al.*, 1990; Rodriguez-Tarduchy *et al.*, 1990; Rolink *et al.*, 1991). Determination of the characteristics which cause these precursors B-cells to be susceptible to apoptosis is a complicated process and is currently under intense investigation by a number of laboratories.

Table 3.1 Plasma corticosterone concentrations and thymus weights after pellet implantation

	Plasma CS* (μg/dl)		Thymus Weight (mg)	
Time	Control‡	CS§	Control‡	CS§
6 hours	20.5±5.0	142.3±30.8†	30.5±3.0	23.6±1.2†
12 hours	23.0±10.2	94.1±11.3†	26.7±2.8	19.0±2.9†
18 hours	12.3±3.0	72.7±21.2†	27.2±4.5	13.6±1.7†
24 hours	7.1±5.9	69.1±24.1†	26.0±1.9	10.6±1.6†
36 hours	11.6±6.4	65.0±13.1†	19.8±3.9	5.5±1.9†
3 days	14.8±10.1	31.3±5.5†	31.2±6.0	5.2±0.6†
5 days	5.8±8.0	37.3±12.5†	26.0±3.6	4.8±0.9†
10 days	14.0±5.0	31.6±5.9†	24.3±3.7	2.5±0.8†
15 days	9.7±8.6	16.8±9.1	29.0±3.6	7.4±5.9†

Data are expressed as mean±SD and represent at least 2 separate experiments. n=4 for data collected through 36 hours. n=7-10 for data collected 3-15 days.

*Plasma CS collected between 8-9a.m.

†significantly different than control, $p<0.05$

‡Sham-control pellet implanted

§CS=corticosterone pellet implanted

Table 3.2 Spleen weight and white blood cell counts in mice after pellet implantation

	Spleen Weight (mg)		WBC/ml blood ($x10^6$)	
Time	Control	CS	Control	CS
5 days	60.3±8.6	27.8±1.3*	3.9±0.7	1.2±0.3*
10 days	61.8±6.1	35.4±4.0*	5.3±0.4	2.0±0.9*

*significantly different than control, $p<0.05$.

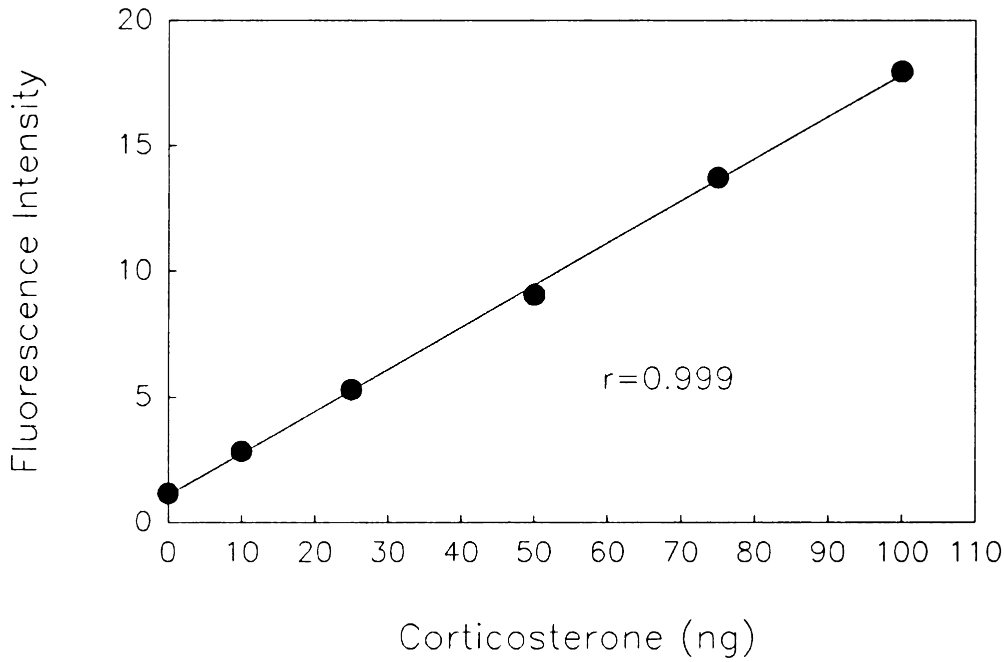
Data are expressed as mean±SD and represent at least 2 different experiments. n=7-10

Table 3.3 Cell cycle distribution of B220$^+$ bone marrow lymphocytes 1 and 5 days after pellet implantation

Treatment	Percentage of B220$^+$ Lymphocytes*		
	G_0/G_1	S	G_2/M
1 day Control	83.7±1.6	13.3±1.7	3.3±0.4
1 day CS	90.7±0.4	3.5±0.4	2.6±0.5
5 days Control	85.7±4.5	10.9±3.7	2.9±0.9
5 days CS	96.1±0.5	0.8±0.1	2.2±0.3

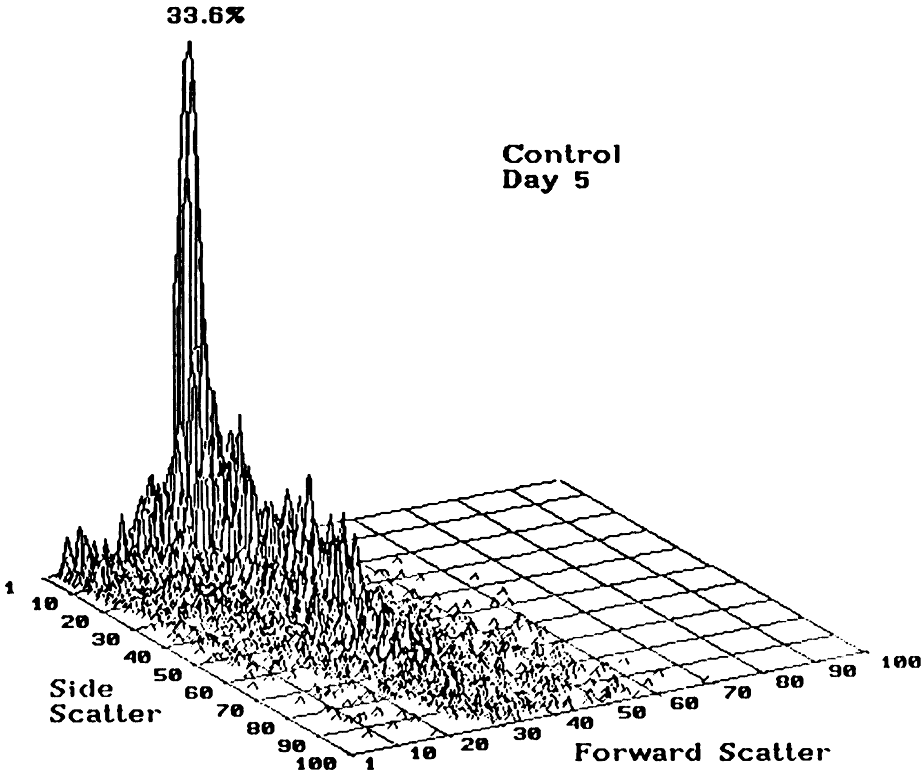
*n=4, data are expressed as mean±SD and are representative of 2 separate experiments.

Figure 3.1 Standard curve for plasma corticosterone assay. Various concentrations of CS were dissolved in 95% ethanol and diluted in dichloromethane. Subsequent to vigorous mixing with 3 parts concentrated H_2SO_4 and 1 part absolute ethanol, fluorescence intensity was measured at 525 nm after excitation at 475 nm. Data points represent the mean of 2 or 3 determinations.



20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
110
Fluorescence Intensity
Corticosterone (ng)
r=0.999

Figure 3.2 Flow cytometric light scatter profiles of bone marrow from control or CS-treated mice 5 days after pellet implantation. Forward scatter on the y-axis is indicative of cell size and side (90°) scatter on the x-axis is indicative of the granular contents of the cell. Percentages are given for the proportion of small, nongranular cells (lymphocytes). Data from a single control or CS-treated mouse is shown representating 8 mice from the same experiment and over 6 separate experiments.


33.6%
Control
Day 5
Side
Scatter
Forward Scatter
1
10
20
30
40
50
60
70
80
90
100

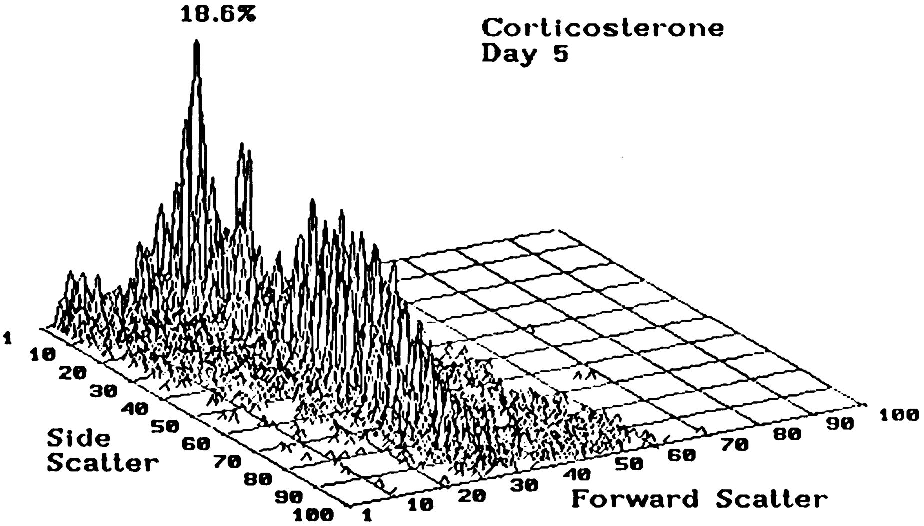

18.6%
Corticosterone
Day 5
Side
Scatter
Forward Scatter
1
10
20
30
40
50
60
70
80
90
100

Figure 3.3 Proportion of B-lineage cells expressing sIg or B220 in whole bone marrow of sham-control or CS-treated mice over a 15 day period. Mean fluorescence intensities measured on a linear scale were obtained from phenotype-positive cells by flow cytometric analysis. Data represent the means (±SD) of 7-10 mice being representative of at least 3 different experiments. Asterisks indicate significant ($p<0.05$) differences from sham controls analyzed on the same day.

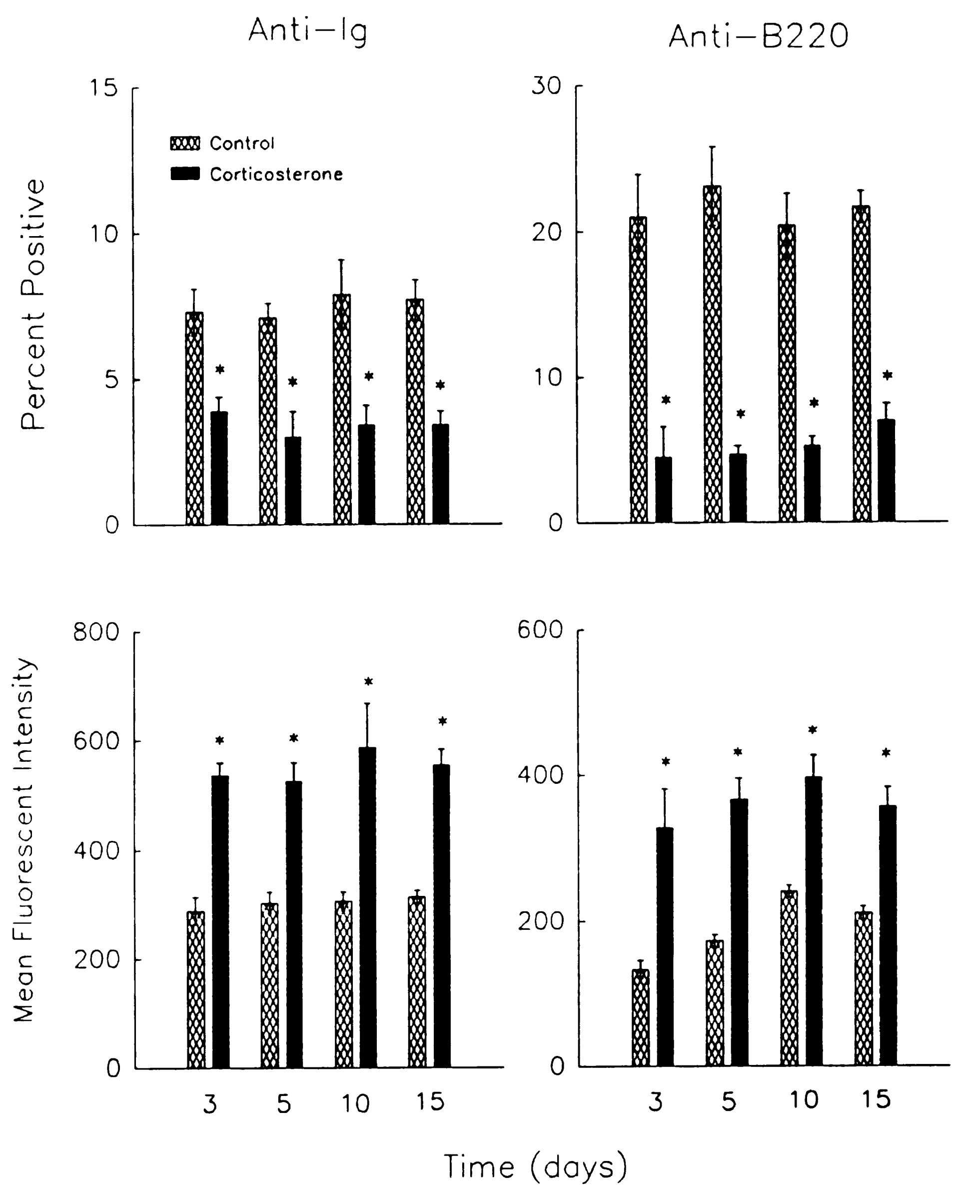

Anti–Ig
Anti–B220
Control
Corticosterone
Percent Positive
Mean Fluorescent Intensity
Time (days)
0
5
10
15
20
30
200
400
600
800
3
5
10
15
*

Figure 3.4 Two-color phenotypic analysis of bone marrow cells 5 days after pellet implantation. Cells were labelled with fluorescent antibodies to B220 and IgM or IgD and IgM. Cells were gated using light scatter profiles to exclude cell debris. Data are plotted as IgM (green fluorescence) on the x-axis versus B220 (red fluorescence) on the y-axis in the two left panels. The two right panels are cytograms representing IgD (green fluorescence) on the x-axis and IgM (red fluorescence) on the y-axis. Region 1 contained cells which were $B220^{+}IgM^{-}$ or $IgM^{+}IgD^{-}$, region 2 cells were $B220^{+}IgM^{+}$ or $IgM^{+}IgD^{+}$, and region 3 contained unlabelled cells. Region 4 contained large, nonlymphoid elements which nonspecifically bound antibody as determined by light scatter profiles. Data were from a single sham-control or CS-implanted mouse representing 8 mice from the same experiment and 4 separate experiments.

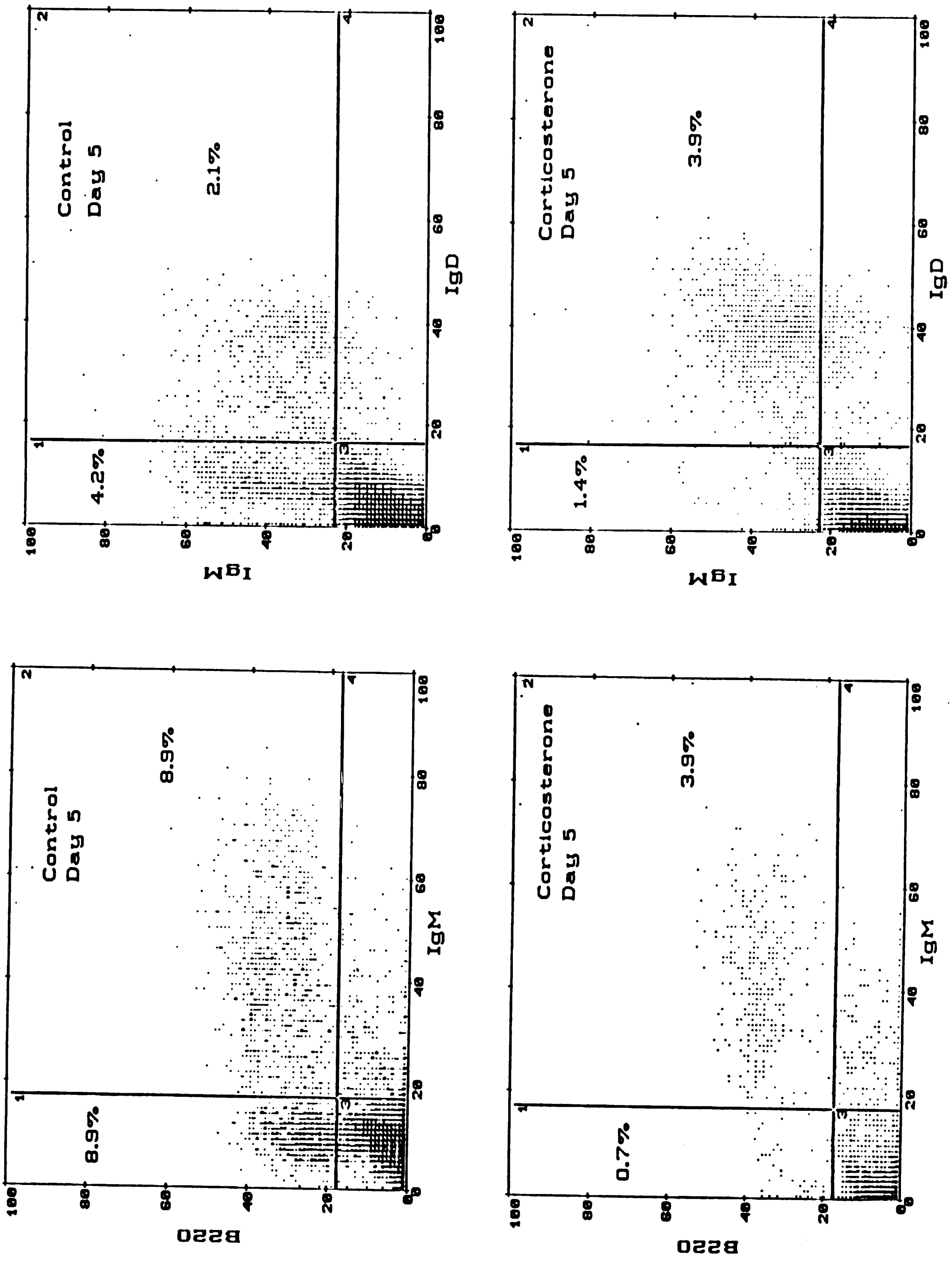

Control
Day 5
2.1%
4.2%
IgD
IgM
Corticosterone
Day 5
3.9%
1.4%
IgD
IgM
Control
Day 5
8.9%
8.9%
IgM
B220
Corticosterone
Day 5
3.9%
0.7%
IgM
B220

Figure 3.5 B220-gated cell cycle histograms of bone marrow 1 and 5 days after pellet implantation. Bone marrow cells from sham control and CS-implanted mice were fluorescently labelled with B220, fixed in 50% ethanol, their DNA stained with propidium iodide (PI), and analyzed using flow cytometry. $B220^+$ cells were gated to exclude debris and cells nonspecifically binding B220 antibody. Data are plotted as $B220^+$ cell number on the x-axis versus PI (red fluorescence) on the y-axis. Phases of the cell cycle are indicated on the histogram of the control. Data are from a single sham control or CS-treated mouse representing 4 mice from the same experiment and 2 separate experiments.

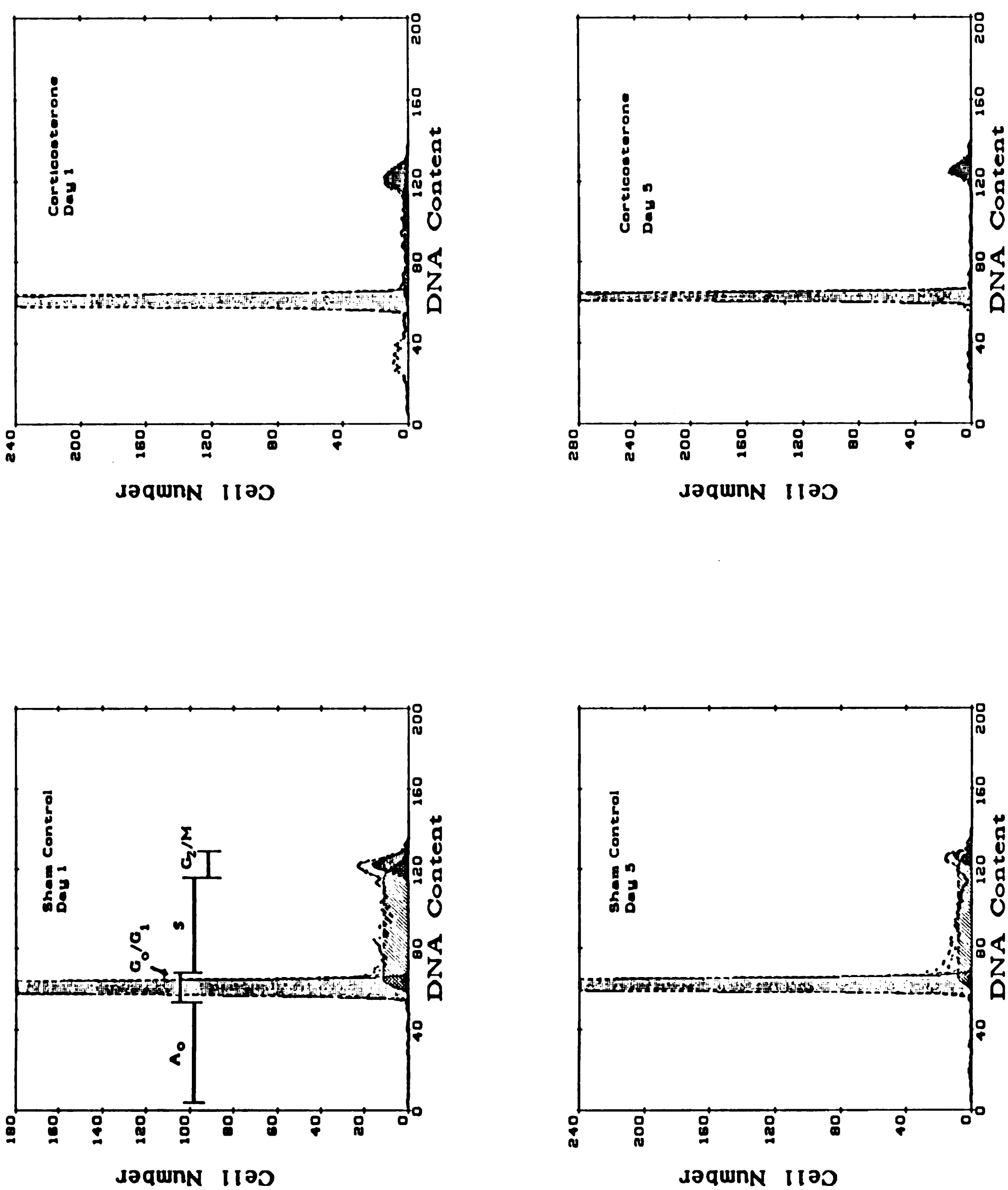
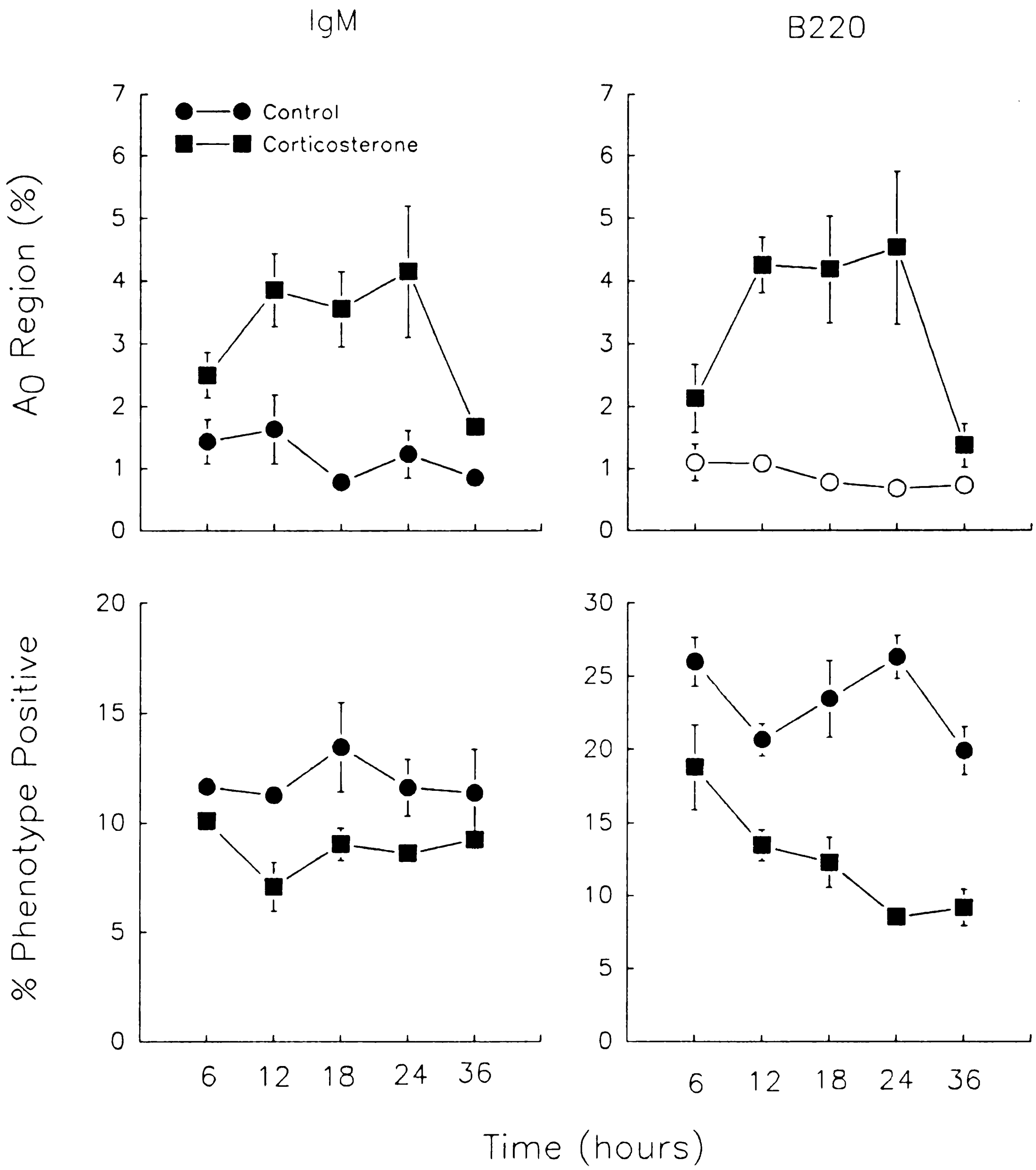

Corticosterone Day 1
Corticosterone Day 5
Sham Control Day 1
Sham Control Day 5
A_0
G_0/G_1
S
G_2/M
Cell Number
DNA Content

Figure 3.6 Induction of apoptosis in conjunction with the change in proportion of B-lineage cells from the bone marrow of CS-treated mice over 36 hours. Top two panels represent the proportion of IgM^+ or $B220^+$ cells in the A_0 region of the cell cycle. Bottom two panels represent the proportion of IgM^+ or $B220^+$ cells in the whole bone marrow. Data are expressed as the mean of 4 mice (±SD) and are representative of 2 separate experiments. Standard deviations are smaller than symbol size at data points with no bars. Significant differences ($p<0.05$) were detected between control and CS-treated mice at all time points except for the proportion of IgM^+ cells in the bone marrow at 36 hours.


IgM
B220
Control
Corticosterone
A0 Region (%)
% Phenotype Positive
Time (hours)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
6
12
18
24
36

Chapter 4

Suppression of the Antigenic Response of Murine Bone Marrow B-cells *In Vitro* by Glucocorticoids

SUMMARY

Data presented herein indicate that the immature B-cells of murine bone marrow (BM) may be as sensitive to glucocorticoids (GC) as are immature thymocytes since physiological levels of the steroids significantly inhibited the response of these cells to trinitrophenylated lipopolysaccharide (TNP-LPS) in short term culture. The *in vitro* response of B-cells of the marrow to TNP-LPS was reduced more than 50% by concentrations of corticosterone (CS) and cortisol (HC) analogous to those found in plasma during stress and trauma. The more potent synthetic GC, dexamethasone (DX), caused a 50-80% decrease in plaque-producing cells at concentration of 10^{-6} and 10^{-8} M. The same pattern of inhibition was noticed regardless of whether DX was added 24 hours prior or up to 48 hours after addition of antigen to culture. However, no inhibition in the response of B-cells was noted when DX was added 72 to 96 hours after stimulation of the cultures. Culture of cells with factor-rich conditioned media did not protect from the DX-induced inhibition of plaque-forming cell production. These effects were found to be specific for GC since neither testosterone nor progesterone at physiological concentrations inhibited the response while the GC receptor antagonist RU 38486 provided protection. A greater than 80% reduction in the proportion of B-cells present in the DX-treated cultures was noted after 5 days corresponding to the 80% inhibition of plaque forming

cell production observed at that time. This reduction in B-cells was rapid since almost 40% of the $B220^+$ cells were depleted within 12 hours of DX addition. These data indicate that physiological levels of GC can readily inhibit the capacity of BM to respond to antigen by depleting the cultures of immature B-cells.

INTRODUCTION

Malnutrition, trauma, surgery and infection all cause a three- to four-fold chronic elevation of plasma GC in both humans and rodents (DePasquale-Jardieu & Fraker, 1980; Kagan *et al.*, 1989). Although the immunosuppressive properties of acute or pharmacological levels of GC have long been recognized (Claman, 1972), there was little information regarding the effects of physiological levels of GC on cells of the immune system, especially the immature and precursor B-cells of the bone marrow (BM). In the latter case, it was known that low concentrations of cortisol (10^{-7} M) inhibited the generation of lymphoid cells but had no effect on the generation of myeloid cells in long term murine BM culture systems (Schrader *et al.*, 1979; Hayashi *et al.*, 1984), although others have found that much higher concentrations inhibit colony-forming units and mononuclear phagocyte growth (Van der Meer *et al.*, 1986). Several injections of pharmacological doses of DX were found to reduce the percentage of BM B-cells by one-half although the residual cells gave a normal response to lipopolysaccharide (LPS) (Sabbele *et al.*, 1987; Benner & Van Oudenaren, 1979). Similar observations have been reported for the murine BM B-cell response to sheep red blood cells after injection of large doses of DX or HC (Benner *et al.*, 1978; Levine & Claman, 1970). Since the latter studies were performed using either pharmacological levels and/or acute exposure to steroids, the specific effects of physiological levels of GC on the function of the immature B-cells of the marrow remained unclear.

In the previous chapter it was reported that the function of BM B-cells resistant to chronic elevation of plasma CS was equal to or greater than that of sham control mice. It should be recognized, however, that those residual cells represented a clearly GC-resistant population of mature B-cells. Experiments were initiated herein to determine whether physiological levels of GC had similar effects on the immature, developing B-cells of murine BM using doses which corresponded to plasma levels found in normal (5×10^{-7} M) and stressed mice (5×10^{-6} M) (DePasquale-Jardieu & Fraker, 1980; Kagan *et al.*, 1989; Besedovsky *et al.*, 1978). A culture system was employed whereby the responses of a specific subset of immature B-cells of the marrow to a hapten-carrier antigen could be monitored in the presence and absence of GC (Medina *et al.*, 1988). It should be pointed out that the experiments contained in this chapter were the initial studies performed in this dissertation project. The short term BM culture system used in these studies had been developed previously in this laboratory and represented a convenient tool for determining if developing B-cells are sensitive to GC.

METHODS

Mice

Male adult A/J mice (Jackson Labs, Bar Harbor, ME) were routinely used at 8-12 weeks of age. In some experiments, C57Bl/6 and CAF_1 mice also were used. They were maintained in a temperature- and light-controlled room and were provided a commercial chow (Purina, St. Louis, MO) with free access to acidified water.

Preparation of Trinitrophenylated Lipopolysaccharide

Trinitrophenol (TNP) was coupled to lipopolysaccharide (LPS) (trichloroacetic acid extracted from E. coli 055:B5) (Difco, Detroit, MI) as previously described (Medina *et al.*, 1988). LPS (25 mg) and trinitrobenzene sulfonic acid (TNBS) (15 mg) were dissoved in 2.5 ml 0.28 M cacodylate buffer (pH 6.9) and adjusted to pH 11.5 with dropwise addition of 2 N NaOH. After stirring at room temperature for 1 hour, the solution was diluted to 5 mg/ml with PBS (pH 7.4) and dialyzed against PBS overnight. After conjugation and dialysis the TNP-LPS solution was sonicated, sterilized by filtration, and stored protected from light at 4°C.

Short Term Bone Marrow Culture

BM cells were prepared for culture using a modification of the method of Medina *et al.* (1988). BM cells were flushed from femurs and tibias of mice and suspended by aspiration into PBS containing 2% fetal calf serum (FCS). Contaminating red blood cells were removed by lysis in 0.16 M Tris containing 0.17 M NH_4Cl. After washing by centrifugation at 400 xg for 5 minutes, the nucleated cells were suspended at a concentration of 5 x 10^5 small nucleated cells/ml in RPMI-1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 5 x 10^{-5} M 2-mercaptoethanol, 5% FCS (M.A. Bioproducts, Walkersville, MD), and 0.5% globulin free bovine serum albumin (Sigma, St. Louis, MO). Aliquots (0.6 ml) of cells were pipetted into 24 well tissue culture plates with 0.01 μg/ml TNP-LPS at 37°C under an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 (%Medina,88%). Steroids were dissolved in 95% ethanol and diluted in RPMI-1640 such that the concentration of ethanol in the cultures never exceeded 0.1%. On average, four replicate wells were used per treatment. One lot of FCS was used throughout which contained a negligible concentration of GC (1.3x10^{-8} M), most of which was bound to transcortin (Brien, 1981). Progesterone, testosterone, cortisol, corticosterone, and dexamethasone were obtained from the Sigma Chemical Co. (St Louis, MO). The GC antagonist, RU 38486, was a gift from Roussel-Uclaf, Romainville, France. In some experiments, cells were cultured without steroid and supernatants obtained at various times by harvesting the cultures and removing the cells by centrifugation.

Supernatants were filtered through 0.22 μm filters for sterilization and stored at -20°C.

Plaque Assay for Quantification of TNP-Responsive Cells

At the optimum point of response (day 5), the number of anti-TNP antibody producing cells was determined using TNP-coated sheep red blood cells (Medina *et al.*, 1988). Sheep red blood cells (SRBC, Colorado Serum) were washed in PBS, pH 7.4, containing 1% (w/v) glucose 3 times by centrifugation at 400 xg for 7 minutes. SRBC (1 ml packed) were incubated for 12 minutes at room temperature with 40 mg TNBS dissolved in 7.5 ml cacodylate buffer. Conjugated cells were washed 3 times in PBS-glucose containing 1% heat-inactivated FCS and suspended in PBS-glucose at a concentration of $2x10^9$ cells/ml. BM cells were removed from 24 well plates by gentile aspiration, pelleted by centrifugation at 400 xg for 5 minutes, and resuspended in 75 μl tris buffered Hanks balanced salt solution, pH 7.2, containing 1% heat-inactivated FCS. BM cells were mixed with 100 μl TNP-coupled SRBC and 1 ml 0.6% agarose in tris buffered minimal essential media (MEM, M.A. Bioproducts, Walkersville, MD) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 56°C. The cell suspension was quickly plated onto previously prepared 60x15 mm polystyrene dishes containing 1.2% agarose in MEM. The plates were incubated at 37°C for 2 hours and plaques developed by incubation for 1 hour at 37°C with 0.5 ml/plate of a 1/10 dilution of nonhemolytic guinea pig complement (Gibco Laboratories, Grand Island, NY). Plates were stored after removal of the complement solution at 4°C until plaques were counted. Each plaque represented a single antibody producing cell. Non-stimulated cells gave negligible plaque numbers. Data were expressed as anti-TNP plaque forming cells (PFC) per 10^7 small nucleated BM cells originally plated. Figure 4.1 shows a summary of the functional assay described here.

Flow Cytometric Analysis of B-cell Subpopulations in Cultured Cells

Cells cultured under the conditions previously described were harvested, centrifuged, and resuspended in cold (4°C) buffer (PBS pH 7.4, 4% heat-inactivated FCS, 0.15% NaN_3) and fluorescently labelled as described in previous chapters. Aliquots of 10^6 cells were labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Tago, Burlingame, CA) or biotinylated B220 purified from RA3.6B2 cell line supernatants. Streptavidin-conjugated FITC (Av-FITC, Tago) was used to fluorescently label cells incubated with B220. Cells were analyzed for positive fluorescence using an Ortho Cytofluorograph 50H fluorescence activated cell sorter (FACS) with a 2150 computer system. FITC was excited with the 488 nm line of an argon laser and emission detected at 530±15 nm interference band pass. Ten thousand events were analyzed per sample. Thymocytes were used as negative controls.

Statistical Analysis

Statistical analysis was performed using analysis of variance. Newman-Keuls post hoc tests were performed to determine between-group differences at $p<0.05$.

RESULTS

Effect of Glucocorticoids on PFC Response

Concentrations of natural and synthetic GC analogous to those found in stressed rodents were added to BM cultures. As little as 10^{-8} M DX reduced anti-TNP PFC production by 70% during the five day culture period (Figure 4.2). At 10^{-6} M DX, anti-TNP PFC production was reduced by 80%. Two other strains of mice (CAF_1 and C57Bl/6) exhibited identical sensitivity to these concentrations of DX indicating the phenomenon was not unique to A/J mice (Figure 4.3). CS, the natural GC in mice (Spackman & Riley, 1978), which has been reported to be much less potent than DX (Munck & Brinck-Johnsen, 1968), nevertheless caused a 70% or greater reduction in anti-TNP PFC production at a concentration of 10^{-7} M, and greater than 90% at 10^{-5} M (Figure 4.2). HC, the predominant human glucocorticoid, also inhibited PFC production at 10^{-7} M by greater than 60% and by 90% at 10^{-5} M (Figure 4.2).

Effect of Delayed Addition of DX to BM Cultures

In order to determine if GC were inhibiting early, intermediate, or late events associated with the antigenic response of immature B-cells and supporting cell types, DX was added to cultures before, simultaneous with, and after addition of antigen. A 24 hour incubation with DX prior to the addition of TNP-LPS caused a reduction in anti-TNP PFC production of 84% and 94% at 10^{-8} M and 10^{-6} M, respectively

(Figure 4.4) which was similar to the results obtained when DX and TNP-LPS were added simultaneously. When DX was added to cultures 24 and 48 hours after the antigen, 10^{-8} and 10^{-6} M DX continued to inhibit PFC production by greater than 70%. Delaying addition of DX for 72 hours had variable effects on PFC production causing a 50% reduction in some experiments while having no effect in others (data not shown). After 72 hours, the cultures became quite resistant to DX so that a 96 hour delay in the addition of DX had no effect on PFC production (Figure 4.4).

Capacity of a Glucocorticoid Antagonist to Block Steroid Inhibition

The glucocorticoid receptor antagonist, RU 38486, was added to the BM cultures to determine if the inhibition of PFC production produced by DX was exerted through the classic GC cytosolic receptor (Moguilewsky & Philibert, 1984). RU 38486 (10^{-6} M) completely blocked inhibition of PFC production by 10^{-8} M DX and significantly protected against 10^{-6} M DX (Figure 4.5).

Specificity of Steroid Inhibition of PFC Response

The specificity of the inhibition of PFC response in short term BM cultures by DX, HC, and CS was determined by adding the non-glucocorticoid steroids, progesterone and testosterone, to the BM cultures. Testosterone failed to have any significant effect on anti-TNP PFC production in the BM when tested at concentrations of 10^{-6} and 10^{-8} M (Figure 4.6). Progesterone suppressed PFC production by about 50% at a concentration of 10^{-6} M but had no effect on PFC production at either 10^{-10} or 10^{-8} M. Though progesterone caused inhibition of anti-TNP PFC production at 10^{-6} M, this inhibition was never greater than that seen with DX and occurred only at what would be considered a pharmacological concentration of this steroid (Schuurs & Verheul, 1990).

Failure of Culture Supernatants to Protect Against DX

Owing to the heterogeneous nature of the BM, it was difficult to determine if GC had a direct effect on B-lineage cells or if they were disrupting the microenvironment and indirectly suppressing the PFC response. This was an important question since it has been shown that stromal cells produce factors necessary for B-cell development (Kincade *et al.*, 1989; Dorshkind, 1990). Supernatants from BM cultures without GC were collected at various time points and used to culture fresh cells with or without 10^{-7} M DX. The supernatants collected at 24 and 48 hours significantly increased the PFC response in cultures activated with TNP-LPS only, indicating that supernatants contained factors which enhanced PFC production (Figure 4.7). However, addition of DX drastically reduced PFC production even in analogously supplemented cultures suggesting that DX had a direct effect on responsive B-cells which growth factors were unable to overide.

Phenotypic Distribution of B-cells After Exposure to DX

Since there was evidence in the literature (and in previous chapters of this dissertation) that GC caused a depletion of B-cells of the BM when administered *in vivo* (or *in vitro*) (Ku & Owen, 1986), BM cultures were analyzed to determine the proportion and phenotypic distribution of $B220^+$ and sIg^+ cells after exposure to DX. In cultures which had not been stimulated with TNP-LPS, 10^{-7} M DX caused a 40% decrease in percent $B220^+$ and $sIgM^+$ cells within 12 hours (Table 4.1). After the normal 5 day culture period, DX-treated cultures stimulated with TNP-LPS contained less than 3% B-cells while stimulated cultures incubated without DX had greater than 25% B-cells present. The latter represents an almost 90% decrease in the B-cell population caused by DX which closely correlated with the 80% inhibition in PFC production observed in these same cultures. These data indicated that the B-cell compartment of cultured BM was depleted by GC.

DISCUSSION

The results presented in this chapter indicate that physiological concentrations of GC are able to significantly inhibit the ability of BM B-cells to respond to antigen. HC, CS, and DX caused about a three-fold reduction in PFC production at 10^{-6} M which corresponded to the serum corticosteroid levels found in nutritionally stressed mice (DePasquale-Jardieu & Fraker, 1980), malnourished children (Alleyne & Young, 1967), and burn patients (Kagan *et al.*, 1989). At 10^{-8} M, DX continued to inhibit PFC production, whereas the natural GC had no significant effect. Previously, DX had been reported to be 25 times more metabolically active than equal molar concentrations of CS and to be 10 times more active than HC based on inhibition of glucose uptake in thymocytes (Munck & Brinck-Johnsen, 1968). However, the higher potency of DX was evident here only at very low concentrations (10^{-8} M) indicating that naturally produced steroids can also have a deleterious effect on immature B-cells.

The GC antagonist, RU 38486, protected the BM cells from the actions of DX suggesting that the inhibitory effects of GC were mediated through the classical GC cytosolic receptor (Chobert *et al.*, 1983). When added in equal molar concentrations, RU 38486 provided partial protection against inhibition of PFC by DX. This is consistent with other studies which have found that when RU 38486 and DX are at equivalent concentrations, DX has only half maximal effects (Chobert *et al.*, 1983; McMillan *et al.*, 1988).

Testosterone and progesterone failed to have an effect on BM PFC production at physiological concentrations. Nevertheless, 10^{-6} M progesterone caused a 50% suppression of PFC production. It should be noted, however, that normal physiological levels of progesterone in mice are highest during pregnancy, being on the order of 1.5×10^{-7} M, so that 10^{-6} M represents a pharmacological level of the steroid (Schuurs & Verheul, 1990). In addition, several studies have shown progesterone to be immunosuppressive at very high concentrations which was not surprising since progesterone is considered to be an anti-GC which weakly binds the GC receptor (McMillan *et al.*, 1988; Roess *et al.*, 1982; Szekeres-Bartho *et al.*, 1989).

These studies clearly show that bone marrow cells are sensitive to the effects of physiological concentrations of GC such that the ability of immature B-cells to respond to antigen is significantly reduced. Because BM is a heterogeneous population of cells it would be difficult to separate direct effects of steroids on the B-cells themselves versus effects on supporting cell types such as stromal cells, macrophages, etc. However, DX was able to significantly inhibit PFC production when added to cultures as late as 48 hours after stimulation suggesting that it was altering the capacity of B-cells to differentiate into antibody-producing cells. With regard to stromal cells (Gimble *et al.*, 1990), it was recently reported that treatment of a stromal cell line with 10^{-7} M cortisol caused adipogenesis but had no effect on production of macrophage-colony stimulating factor, inducibility of IL-6 mRNA expression, or ability to support proliferation of stromal-cell-dependent B-cell lines. In addition, in experiments in which BM cells were cultured in factor-rich conditioned media, DX still inhibited PFC production. Though not definitive, these studies collectively suggest that the capacity of supporting cells to provide essential factors for B-cell growth and maturation may not be as significantly altered by GC as the B-cells themselves.

Furthermore, the data presented here also is consistent with recent work which suggested that GC were able to suppress the proliferative response of peripheral B-cells to anti-Ig antibody (Bowen & Fauci, 1984; Dennis *et al.*, 1987; Luster *et al.*, 1988) by blocking their entry into the cell cycle. GC also inhibited anti-Ig-stimulated peripheral murine and human B-cells if added to cultures within 24 to 48 hours of stimulation (Bowen & Facui, 1984; Luster *et al.*, 1988). This is analogous to findings herein, since DX was able to significantly inhibit anti-TNP PFC production in BM B-cells only if added within 48 hours of antigen stimulation.

The ability of DX and other GC to inhibit PFC production when added within 48 hours of antigenic stimulation, along with the observed depletion in actual numbers of pre- and immature B-cells created by the steroids, is analogous to the observation that these steroids can inhibit and eliminate immature thymocytes (Compton & Cidlowski, 1986; Umansky *et al.*, 1981). Furthermore, the data presented in the previous two chapters of this dissertation suggest that the loss of cells early in the cultures here were eliminated by induction of apoptosis. The inability of DX to significantly inhibit PFC production after 72 hours also appears to be consistent with the observation that activated B-cells or plasmacytes are in some cases resistant to GC (Dennis *et al.*, 1987). In this regard, it is interesting to note that activated cells have been shown to be more resistant to programmed cell death unactivated cells (Liu *et al.*, 1989).

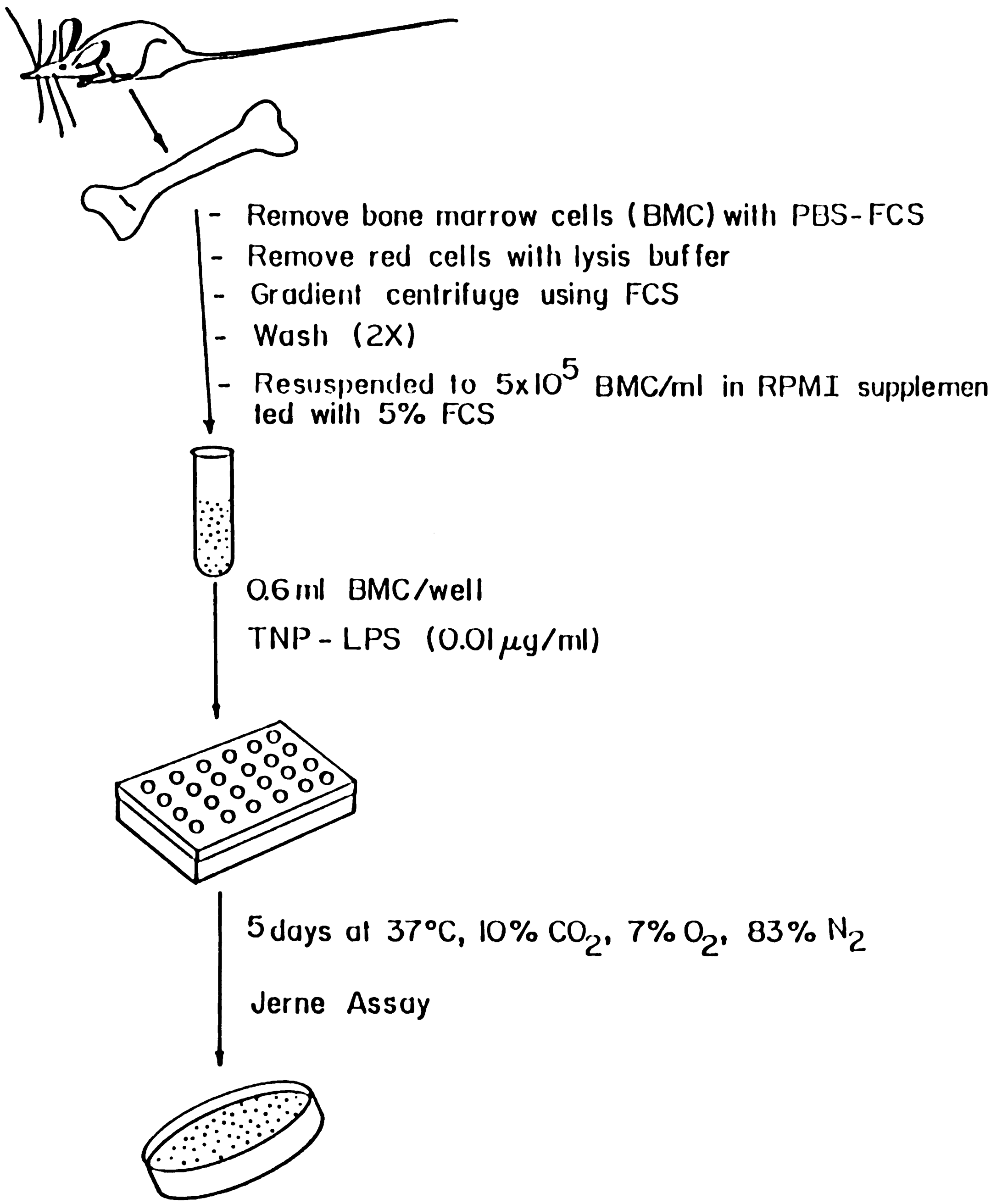
Table 4.1 Phenotypic distribution of sIg^+ or $B220^+$ cells of bone marrow cultured in the presence of dexamethasone with or without TNP-LPS.

		% sIg^+		% $B220^+$	
Time	TNP-LPS	Control	DX	Control	DX
0	-	9.1	--	20.6	--
12 hours*	-	9.7	4.2	21.9	8.2
24 hours	-	10.3	2.5	18.7	4.5
48 hours	-	10.8	1.7	16.2	3.4
5 days†	+	25.6	2.5	29.9	2.9

*Cells were labelled with goat anti-mouse IgM or rat anti-mouse B220.

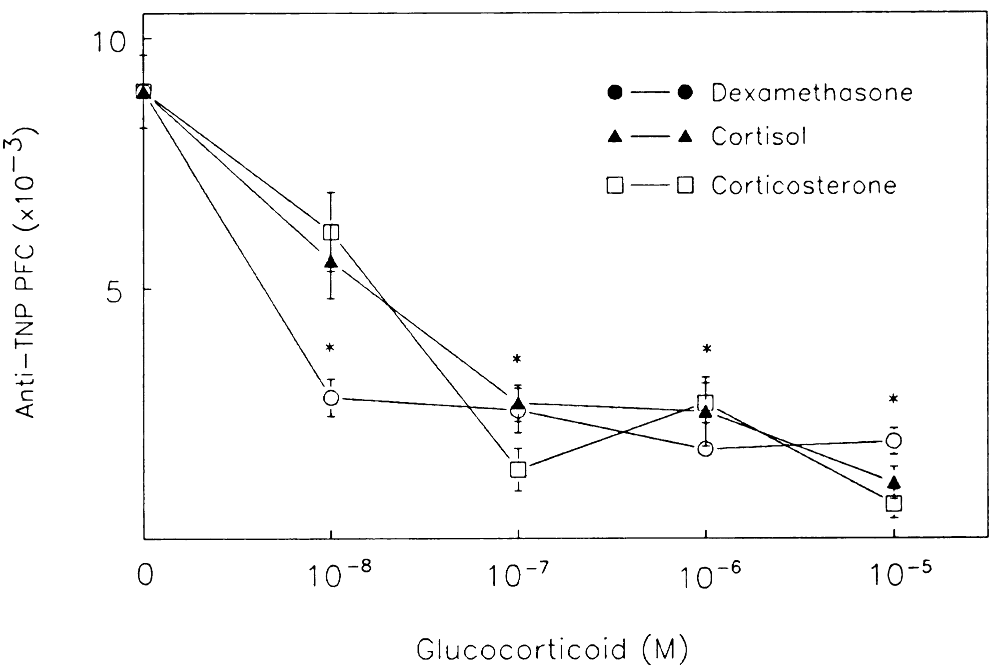
†Cells were labelled with goat anti-mouse Ig or rat anti-mouse B220.

Figure 4.1 Schematic representation of the short term BM culture system used to determine the functional capacity of immature BM B-cells.



- Remove bone marrow cells (BMC) with PBS-FCS
- Remove red cells with lysis buffer
- Gradient centrifuge using FCS
- Wash (2X)
- Resuspended to 5×10^5 BMC/ml in RPMI supplemented with 5% FCS
0.6 ml BMC/well
TNP-LPS (0.01 μg/ml)
5 days at 37°C, 10% CO_2, 7% O_2, 83% N_2
Jerne Assay

Figure 4.2 Day 5 response to TNP-LPS of bone marrow cultures incubated with dexamethasone, corticosterone, or cortisol. Cells were cultured with 0.01 μg/ml TNP-LPS and the indicated concentrations of glucocorticoids. Each point represents the mean (±SEM) number of anti-TNP PFC of 4 cultures. All data with asterisks are significantly ($p<0.05$) different from control cultures with no GC as determined by Newman-Keuls post-hoc tests. Data are representative of 3 separate experiments.



Dexamethasone
Cortisol
Corticosterone
Anti-TNP PFC (x10^{-3})
10
5
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Glucocorticoid (M)

Figure 4.3 Response of bone marrow from three different murine strains to TNP-LPS in the presense of varying concentrations of DX. Data are expressed as the mean ± SEM of 4 cultures and are representative of 2 separate experiments. Points below asterisks are significantly ($p<0.05$) different from cultures without steroid as determined by Newman-Keuls post-hoc tests.

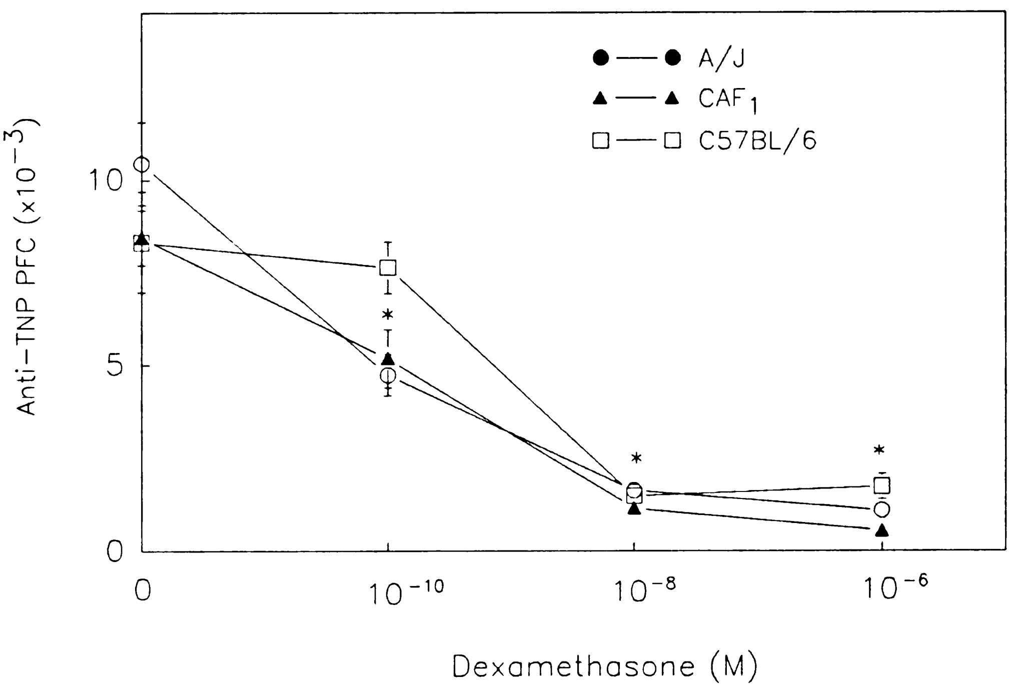

A/J
CAF1
C57BL/6
Anti-TNP PFC (x10^-3)
10
5
0
0
10^-10
10^-8
10^-6
Dexamethasone (M)

Figure 4.4 Time course of effects of dexamethasone on BM PFC response to TNP-LPS. Various concentrations of DX were added to cultures at various times before and after addition of antigen as indicated on the panels. Anti-TNP-PFC production was determined at day 5. Each point represents the mean (±SEM) of 4 cultures. Asterisks indicate significant ($p<0.05$) difference in PFC production from control cultures with no DX as determined by Newman-Keuls post-hoc tests. Some symbols do not have visible error bars since the SEM was very small. Each graph is representative of 3 separate experiments.

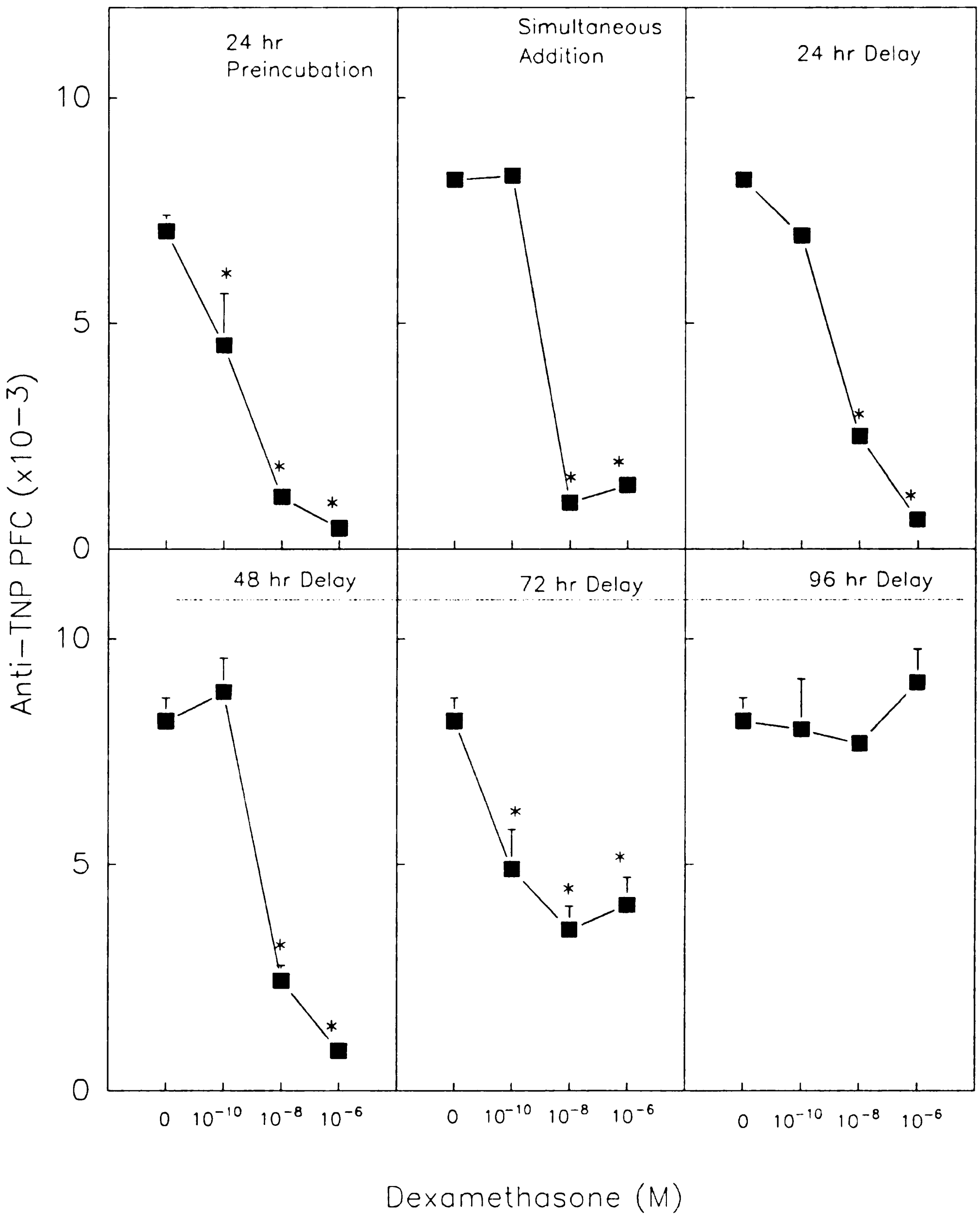

24 hr Preincubation
Simultaneous Addition
24 hr Delay
48 hr Delay
72 hr Delay
96 hr Delay
Anti-TNP PFC (x10-3)
10
5
0
0
10^-10
10^-8
10^-6
Dexamethasone (M)

Figure 4.5 Abrogation of DX-induced inhibition of anti-TNP PFC production by RU 38486. Dexamethasone and the GC receptor antagonist RU 38486 were added to cultures along with 0.01μg/ml TNP-LPS and PFC production was determined after a 5 day incubation. Data are expressed as mean (±SEM) anti-TNP PFC for 4 replicate cultures. Asterisks indicate a significant ($p<0.05$) difference in PFC production from cultures without DX or RU 38486 as determined by Newman-Keuls post-hoc tests. These data are representative of 3 separate experiments.

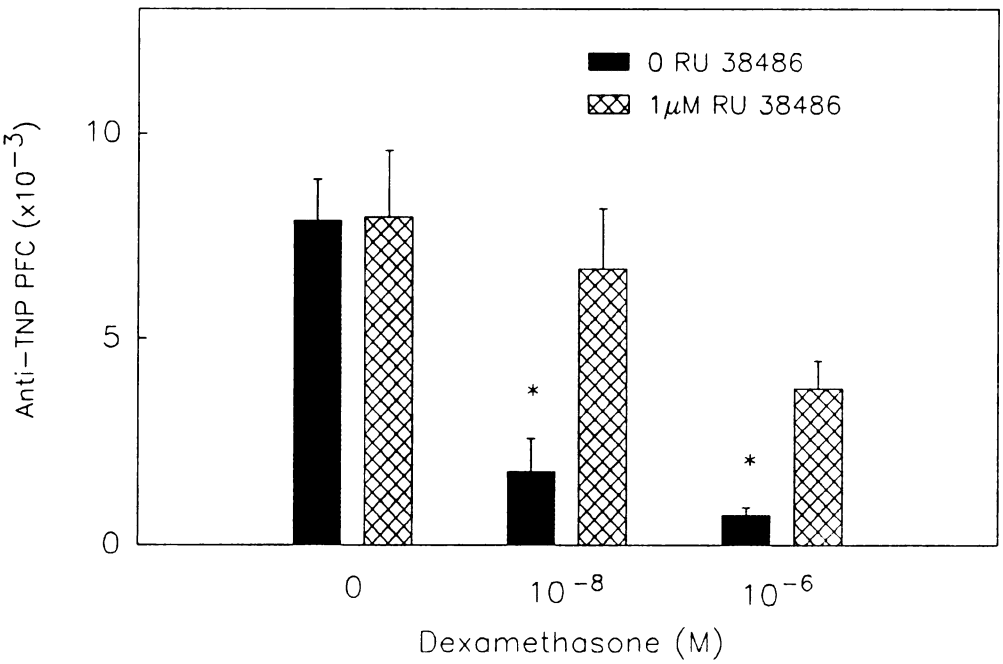
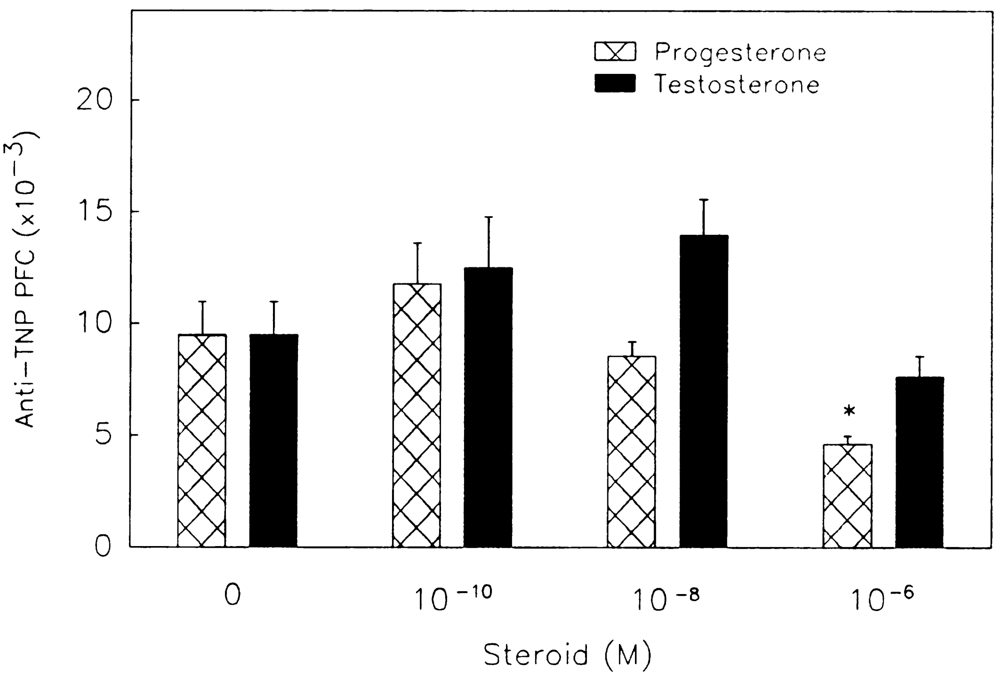
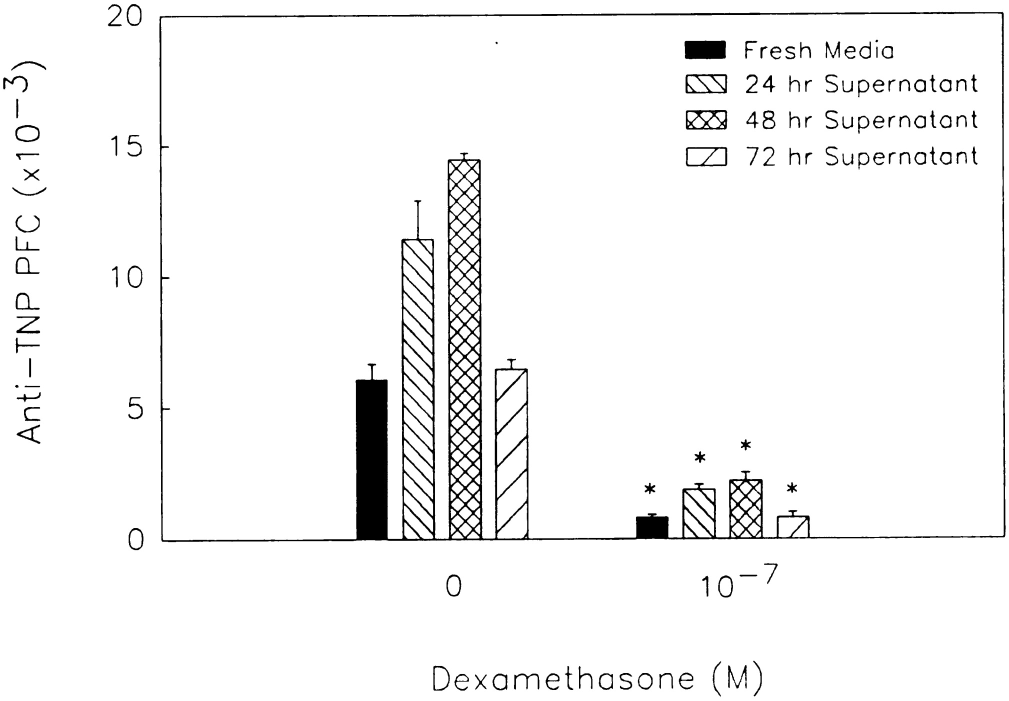

0 RU 38486
1μM RU 38486
Anti−TNP PFC ($x10^{-3}$)
10
5
0
*
*
0
10^{-8}
10^{-6}
Dexamethasone (M)

Figure 4.6 Day 5 response to TNP-LPS of bone marrow cultures incubated with testosterone or progesterone. Cultures were incubated with 0.01μg/ml TNP-LPS in the presence of various concentrations of non-glucocorticoid steroids and anti-TNP PFC production determined at 5 days. Data are expressed as the mean ± SEM of 4 cultures and are representative of 3 separate experiments. Asterisks represent significant ($p<0.05$) differences from control cultures without steroids as determined by Newman-Keuls post-hoc tests.



Progesterone
Testosterone
20
15
10
5
0
Anti-TNP PFC (x10^-3)
*
0
10^-10
10^-8
10^-6
Steroid (M)

Figure 4.7 Day 5 response to TNP-LPS of DX treated bone marrow cultures in conditioned supernatants provided from normal bone cultures. To generate supernatants enriched with growth factors, normal BM cells were incubated with 0.01 μg/ml TNP-LPS and supernatants were collected 24, 48, or 72 hours later. Fresh BM cells were cultured with the supernatants in the presence or absense of 10^{-7} M DX. Data are expressed as the mean ± SEM of 4 cultures and are representative of 2 separate experiments. Asterisks represent significant ($p<0.05$) differences from cultures without steroids.


20
15
10
5
0
Anti-TNP PFC (x10^{-3})
Fresh Media
24 hr Supernatant
48 hr Supernatant
72 hr Supernatant
*
0
10^{-7}
Dexamethasone (M)

Chapter 5

Detection of Glucocorticoid Receptors in B-Lineage Cells

SUMMARY

B-lineage B220$^+$ cells were isolated from bone marrow (BM) using a "panning" method and assayed for the presence of glucocorticoid receptors (GcR) using radiolabelled ligand. Comparison of specific binding of ^{3}H-dexamethasone to B220$^+$ bone marrow cells with that of thymocytes revealed that thymocytes bound a greater amount of radioactivity per 10^6 cells (848 ± 64 DPM) than did B220$^+$ cells (506 ± 180 DPM). Either a 1000-fold excess of unlabelled DX or a 100-fold excess of RU 38486, a GcR antagonist, were able to compete with ^{3}H-dexamethasone for receptor binding sites. Using these limited data, an approximate number of GcR per cell was calculated from the specific binding of a saturating concentration of ^{3}H-dexamethasone (50 nM). Unfortunately, insufficient numbers of B220$^+$ cells could be isolated from the BM for saturation binding and Scatchard analysis. The single concentration estimates revealed that B220$^+$ cells contained approximately 3700 ± 1300 receptors/cell compared to 6200 ± 47 receptors/cell calculated for thymocytes. These values were of the same order of magnitude as receptors/cell reported in the literature for murine lymphocytes (5000-6000). GcR analysis of a chemically transformed cell line, 70Z/3 which has been reported to have a phenotype similar to pre-B cells, indicated that cells growing in mid-log phase contained on the order of 16,000 receptors/cell and a K_d of 17 nM. These data indicated that freshly isolated BM B-lineage cells not only

had GcR but they had similar numbers of GcRs as thymocytes.

INTRODUCTION

The cellular effects of GC are mediated by a cytoplasmic receptor which, upon ligand binding, translocates to the nucleus and alters transcription by its interaction with a specific DNA recognition site. The GcR of thymocytes, splenocytes, and lymph node cells from a variety of species have been analyzed fairly thoroughly (Ranelletti *et al.*, 1981; Duval *et al.*, 1976; Duval *et al.*, 1979; Martins *et al.*, 1987; Armanini *et al.*, 1988; Schlechte *et al.*, 1982; Crabtree *et al.*, 1980). Estimation of the number of GcRs per cell range between 1000 and 7000 in normal lymphocytes with dissociation constants (K_d) on the order of 1-40 nM (Crabtree *et al.*, 1980; Duval *et al.*, 1976; Armanini *et al.*, 1988).

Dieken *et al.* (1990) suggested that the N-terminal immunomodulatory domain of the GcR was essential for induction of apoptosis in murine S49 lymphoma cells since they found mutants which lacked most of the immunomodulatory domain were resistant to GC-induced apoptosis. Further, Harbour *et al.* (1990) found that the DNA binding domain of the GcR was essential for DX-mediated lysis of human T-leukemic cells. Transfection of mutants lacking GcR with constructs containing functional ligand and DNA binding domains caused cell death while DX had no effect on viability of cells transfected with constructs lacking a functional DNA binding region.

In the previous chapters of this dissertation, it was shown that B-lineage cells underwent apoptosis when exposed to physiological concentrations of GC both *in vitro* and *in vivo*. Indirect evidence using a well-characterized GcR antagonist, RU 38486 (Moguilewsky & Philibert, 1984; Chobert *et al.*, 1983), suggested that GC-induced apoptosis was mediated by the classical GcR since RU 38486 provided complete protection (see Figure 2.7). Since the GcR appears to be a critical mediator in the effects of GC on lymphocytes, it was important to confirm the presence of GcRs in BM B-lineage cells. This analysis was complicated by the heterogeneous nature of the BM and the difficulty of obtaining pure subsets of B-cells. Therefore, after developing methods for fractionating BM into $B220^+$ and $B220^-$ populations, a saturating concentration of radiolabelled DX was used to estimate GcR concentrations in $B220^+$ BM cells and thymocytes. These results demonstrate that $B220^+$ BM cells have GcRs; however, limited availability of cells prevented saturation-binding/Scatchard plot analysis. $B220^+$ cells were chosen for study since these cells represented 20-30% of total BM and, unlike IgM^+ cells, could be obtained in high enough numbers to do the desired analyses.

METHODS

Absorption of Glucocorticoids from Fetal Calf Serum

GC found in fetal calf serum (FCS) have been shown to cause translocation of the GcR from the cytoplasm to the nucleus (Picard & Yamamoto, 1987). To minimize the possible interference of such steroids in the receptor binding assays described below, FCS was treated with dextran-coated charcoal which reportedly removes GC from serum (Hayashi *et al.*, 1984). FCS (M.A. Bioproducts, Rockville, MD) was mixed with 1 mg/ml dextran (average MW 38,800, Sigma Chemical Co., St. Louis, MO) and 10 mg/ml Norit A activated charcoal (Matheson Coleman & Bell, Norwood, OH) and incubated for 30 minutes in a 56°C water bath with frequent shaking. Dextran-coated charcoal was removed from FCS by centrifugation at 12,000 xg for 10 minutes at 4°C, filtered through a 0.22 μm filter and stored at -20°C. Dextran/charcoal-treated FCS was used in all buffers and medias described in this chapter.

Preparation of Plates for "Panning"

Rat anti-B220 antibody (IgM) from the RA3.3A1 cell line (ATCC, Rockville, MD) was raised as ascites in sublethally irradiated CAF_1 mice and stored at -70°C without further processing. Raw ascites was diluted 1:100 in Tris buffer, pH 9.5, and 10 ml of antibody solution incubated in 100x15 mm polystyrene petri dishes for 2 hours at room temperature. After removing the antibody solution and washing the plates by gentle rinsing with 4 volumes of PBS, pH 7.4, the plates were incubated for 1 hour with 10 ml PBS, pH 7.4, supplemented with 0.2% (w/v) nonfat dry milk (Carnation Co., Los Angeles, CA) which was found to efficiently block nonspecific binding sites. The plates were kept at room temperature until the addition of cell suspensions.

Fractionation of B220$^+$ Cells from Murine Bone Marrow

BM cells from A/J mice were fractionated into B220$^+$ and B220$^-$ populations using a modification of the method of Wysocki and Sato (Wysocki & Sato, 1978). BM was flushed from femurs and tibias of A/J mice (The Jackson Laboratories, Bar Harbor, ME) with PBS, pH 7.4, supplemented with 2% heat-inactivated FCS and red blood cells removed by centrifugation through Histopaque 1083 (Sigma Chemical Co., St. Louis, MO). The cells were washed twice by centrifugation at 400 xg for 5 minutes and suspended at a concentration of $2x10^6$ cells/ml in PBS with 5% heat-inactivated FCS. Anti-B220-coated plates were incubated with 5 ml of the cell suspension for 1 hour at 4°C with gentle swirling after 30 minutes. Nonadherent cells were removed from the plates by washing with 4-5 volumes of PBS. Microscopic inspection determined when the majority of nonadherent cells had been removed. Plates with the adherent cells were incubated for 1 hour at 37°C under an atmosphere of 10% CO_2, 7% O_2, 83% N_2 in 7 ml RPMI-1640 medium without phenol red (M.A. Bioproducts, Rockville, MD), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 50 μM mercaptoethanol, and 5% heat-inactivated FCS. Adherent cells were removed from plates by vigourous pipetting in PBS supplemented with 2% FCS. Microscopic inspection indicated when the maximum number of cells had been recovered. Pipets and tubes used for recovery of adherent cells were pre-coated with the PBS/milk solution to minimize losses of cells. The recovered adherent cells were about 15% of the whole BM loaded onto the plates and were greater than 90% viable as determined by trypan blue exclusion. The adherent population was consistently greater than 80% B220$^+$ cells when analyzed using fluorescently labelled anti-B220 and flow cytometry as described in Chapter 2.

Whole Analysis of Glucocorticoid Receptor Binding to ^{3}H-Dexamethasone

Thymuses were removed, minced, and passed through a 100 mesh screen to obtain a single-cell supension. BM was flushed from femurs and tibias. Red blood cells were removed by centrifugation through Histopaque 1086 followed by two washes with PBS and 2% FCS. Thymocytes, whole BM cells, or B220$^+$ BM cells were pelleted by centrifugation at 400 xg for 5 minutes and suspended at a concentration of 10^6 cells/ml in RPMI-1640 without phenol red, but supplemented as described above without FCS. Aliquots of 1 ml cell suspensions were pipetted into 1.5 ml eppendorf tubes. Total binding was determined by adding ^{3}H-1,2,7-dexamethasone (specific activity 37 Ci/mmol, Amersham, Arlington Heights, IL) diluted in 95% ethanol to the appropriate tubes in 10 μl aliquots to give final ^{3}H-DX concentrations as indicated in Figures 5.1-5.3. Nonspecific binding was determined by adding ^{3}H-DX plus a 500 to 1000-fold excess of unlabelled DX or a 100-fold excess of RU 38486 dissolved and diluted in 95% ethanol and added to appropriate tubes in 10 μl aliquots. Cells were incubated at 37°C for 45 minutes, pelleted by centrifugation at 400 xg for 5 minutes at 4°C, and washed 4 times with 1-ml volumes of ice cold PBS, pH 7.4, followed by centrifugation at 400 xg for 5 minutes. Incubation time and the number of washes needed to free the cells of unbound radioactivity was optimized as shown in the results section. After the final wash, 1 ml of Safety Solve (Research Products International Corp., Mount Prospect, IL) was added to the tubes to solubilize the cells and the tube contents were transfered to scintillation vials. Eppendorf tubes were rinsed with 1 ml 95% ethanol which also was transferred to scintillation vials. The vials were filled with 5 ml Safety Solve and radioactivity was quantitated on a Packard scintillation counter. Cell samples with no radioctivity added were run for determination of background DPM and subtracted from the experimental samples.

Glucocorticoid Receptor Analysis of the 70Z/3 Cell Line

70Z/3 cells (ATCC, Rockville, MD), used for receptor binding studies, were maintained at 37°C in RPMI-1640 medium without phenol red supplemented with 2 mM glutamine, $5x10^{-5}$ M mercaptoethanol, and 10% FCS under an atmosphere of 5% CO_2 in air. For receptor binding assays, the cells were removed from culture during the mid-log phase of growth, centrifuged at 400 xg for 7 minutes, and suspended at 10^6 cells/ml in RPMI-1640 medium without phenol red as described above. GcR analysis was carried out using the whole cell binding assay described above.

RESULTS

Determination of the Number of Washes and Optimal Time of Binding in the Whole-Cell Glucocorticoid Receptor Assay

In order to determine the number of washes necessary to remove non-bound radioactivity, BM cells were incubated at 37°C for 1 hour with 10 nM ^{3}H-DX with or without 5 μM of unlabelled DX and washed up to 6 times with 1-ml volumes of ice cold PBS before scintillation counting. Total and nonspecific binding decreased with successive washes until no further decrease in ^{3}H-DX bound was observed (Figure 5.1). In all subsequent experiments, 4 washes were performed before scintillation counting.

The length of time required for maximal binding of ^{3}H-DX to the intracellular receptor at 37°C was determined by incubating unfractionated BM cells with 10 nM ^{3}H-DX with or without 5 μM of unlabelled DX. Maximal total and nonspecific binding occured within 10 minutes of incubation and remained constant throughout 1 hour (Figure 5.2). Specific binding, determined by subtracting nonspecific from total binding, remained fairly constant between 10 and 60 minutes of incubation. In subsequent experiments, BM-derived cells were incubated for 45 minutes at 37°C to ensure that maximal binding had taken place. Since similar kinetics have been reported for murine thymocytes (Duval *et al.*, 1979), incubation periods for thymocytes in subsequent experiments were also 45 minutes.

Analysis of ^{3}H-dexamethasone Binding to B220^{+} Bone Marrow: Comparison to Thymocytes

Due to the low number of B220^{+} cells in the BM, it was not possible to obtain enough viable cells at one time to perform ^{3}H-DX saturation binding experiments and subsequent Scatchard analyses. Therefore, we estimated the total number of GcR per cell by measuring the amount of ^{3}H-DX specific binding at a saturating concentration (50 nM) of ^{3}H-DX and compared the amount found in B220^{+} cells to that in thymocytes (which contain a known number of GcR per cell). Since the K_d reported for murine lymphocytes has been reported to be on the order of 1-40 nM (Dausse *et al.*, 1977; Duval *et al.*, 1976; Duval *et al.*, 1979), 50 nM $^{+}$H-DX was chosen as the ligand concentration for GcR saturation. Specific binding was determined by subtracting nonspecific binding (cells incubated with ^{3}H-DX plus 1000 fold excess DX) from total binding (cells incubated with ^{3}H-DX alone). Specific binding of ^{3}H-DX was 506 ± 180 DPM/10^{6} cells for BM B220^{+} cells compared to 848 ± 64 DPM/10^{6} for thymocytes (Table 5.1). An estimate of the number of binding sites per cell revealed that B220^{+} BM cells had on the order of 3700, while thymocytes had around 6200 sites/cell (Table 5.1). These values were comparable to published reports for murine thymocytes and splenocytes as shown in Table 5.1. Further, similar numbers of specific ^{3}H-DX binding sites were observed when a 100-fold molar excess of RU 38486, a GcR antagonist, was added as the competitor (data not shown). These data demonstrate similar GcR concentrations in BM B-lineage cells compared to thymocytes.

Determination of Specific Binding Sites and K_d of 70Z/3 Cells

70Z/3 cells are a chemically transformed murine B-cell line which are phenotypically similar to pre-B cells (Paige *et al.*, 1978). These cells express surface B220 and are sIg^- but will express sIgM when induced by LPS, dextran sulfate, or IL-1 (Paige *et al.*, 1978; Bomsztyk *et al.*, 1990). Optimization of the whole-cell binding assay conditions were performed using 70Z/3 cells and found to be identical to that determined for BM (data not shown). Saturation binding of ^{3}H-DX to 70Z/3 cells for three separate experiments and a Scatchard plot representing the combination of the experiments are shown in Figure 5.3. The mean K_d of the three experiments was 17.5 ± 7.1 nM and was comparable to the values reported for other transformed cell lines (Bourgeois & Newby, 1979; Lippman *et al.*, 1974). The number of binding sites was estimated to be 16,400 ± 3700 per cell, 3-4 times higher than that found in normal lymphocytes, but similar to other transformed cell lines (Bourgeois & Newby, 1979).

DISCUSSION

The results presented in this chapter clearly demonstrate the presence of GcR in BM B-lineage cells at concentrations somewhat lower than those in other lymphoid cells. Since limited numbers of B220$^+$ cells could be obtained from BM it was not possible to determine the dissociation constant and the absolute number of receptors per cell. However, the specific binding of ^{3}H-DX to B220$^+$ cells (at estimated saturation) compared favorably with that of thymocytes, which were GcR positive control cells. It was also found that unlabelled DX and RU 38486, a GcR antagonist, competed to a similar degree for ^{3}H-DX specific binding sites in these cells. These data indicated that the GcR found in B220$^+$ cells was comparable, at least in number and apparent binding characteristics, to that found in thymocytes.

Interestingly, a transformed pre-B cell line, 70Z/3, was shown to have four times the number of receptors per cell as normal B-lineage cells. This is not unusual for transformed cell lines since the murine thymoma, WEHI-7, has been reported to have over 25,000 receptors per cell with a K_d of 14 nM (Bourgeois & Newby, 1979). Thus, it appears that, at least in this respect, these cell lines may not be ideal models to substitute for normal B-lineage cells. The higher number of receptors/cell reported for transformed cell lines may be due to the fact that a large percentage of such cells are actively cycling and the number of GcR have been reported by several investigators to vary during progression through the cell cycle (Cidlowski & Cidlowski, 1982; Smith *et al.*, 1977).

Although they have four times more GcR per cell than normal B-lineage cells, preliminary data from this laboratory nevertheless indicates that 70Z/3 cells are not susceptible to GC-induced apoptosis (unpublished data, not shown). This observation supports the idea that the number of receptors a cell has does not always correspond to a greater GC sensitivity although this is a controversial point. Bourgeois and Newby (1977; 1979) reported a high correlation between GcR content and sensitivity to cytolysis of murine thymoma clones. Other investigators, however, report little difference in GcR numbers for the highly sensitive cortical thymocytes versus GC resistant medullary T-cells (Duval *et al.*, 1976; Ranelletti *et al.*, 1981). The differences in GC sensitivity may be more fundamental, may be at the transcriptional level, and thus may not be influenced by the number of GcR. This provides further evidence that transformed cell lines may vary from normal cells with regard to responses to GC.

The data presented in this chapter have shown that normal B-lineage cells possess GcR suggesting that they may be directly regulated by GC. Since it has been shown that functional GcR are necessary for GC induced apoptosis (Dieken *et al.*, 1990; Harbour *et al.*, 1990), the present data further support the proposal that GC directly induced the apoptosis in BM B-lineage cells reported earlier in Chapters 2 and 3.

Table 5.1 Specific binding of ^{3}H-dexamethasone to glucocorticoid receptors of B220$^+$ bone marrow cells and thymocytes; comparison to literature values.

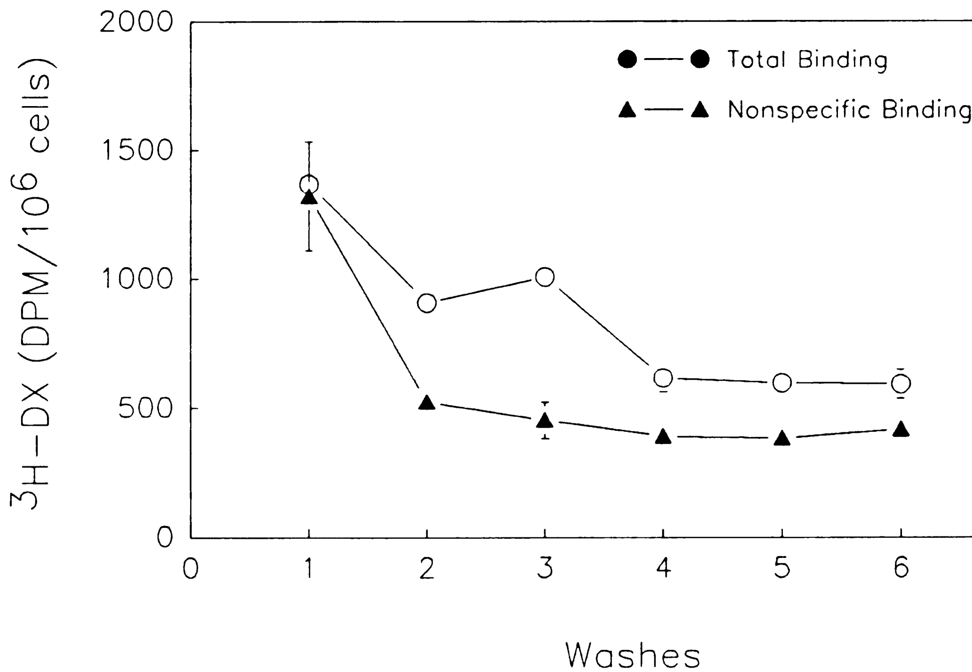
	Specific Binding DPM/10^6 cells†	Receptors/ Cell
B220$^+$ BM cells	506 ± 180*	3700 ± 1300
Thymocytes	848 ± 64	6200 ± 470

	Published Literature Values	Reference
Thymocytes	6000	Dausse *et al.* 1977
	6000	Duval *et al.* 1976
Splenocytes	5500	Duval *et al.* 1979

†A saturating concentration of ^{3}H-dexamethasone with or without 1000-fold excess unlabelled dexamethasone was used to determine specific binding.

*Data are expressed as the mean ± SD.

Figure 5.1 Optimal number of washes for removal of non-specifically bound ^{3}H-dexamethasone. Cells were incubated in 10 nM ^{3}H-DX with or without 500-fold excess unlabelled DX and washed the indicated number of times with 1 ml volumes of ice cold buffer. Data represents the mean ± SD of 3 samples and is representative of 2 different experiments. Symbols without error bars had standard deviations smaller than the symbol size.



2000
1500
1000
500
0
0
1
2
3
4
5
6
7
Total Binding
Nonspecific Binding
^{3}H-DX (DPM/10^6 cells)
Washes

Figure 5.2 Optimal time of binding required for saturation of specific binding sites. Cells were incubated in 10 nM ^{3}H-dexamethasone with or without a 500-fold excess unlabelled DX for the indicated times. Total and nonspecific binding data points represent the mean ± SD of 3 samples and are representative of 2 separate experiments. Specific binding points are the difference between total and nonspecific binding.

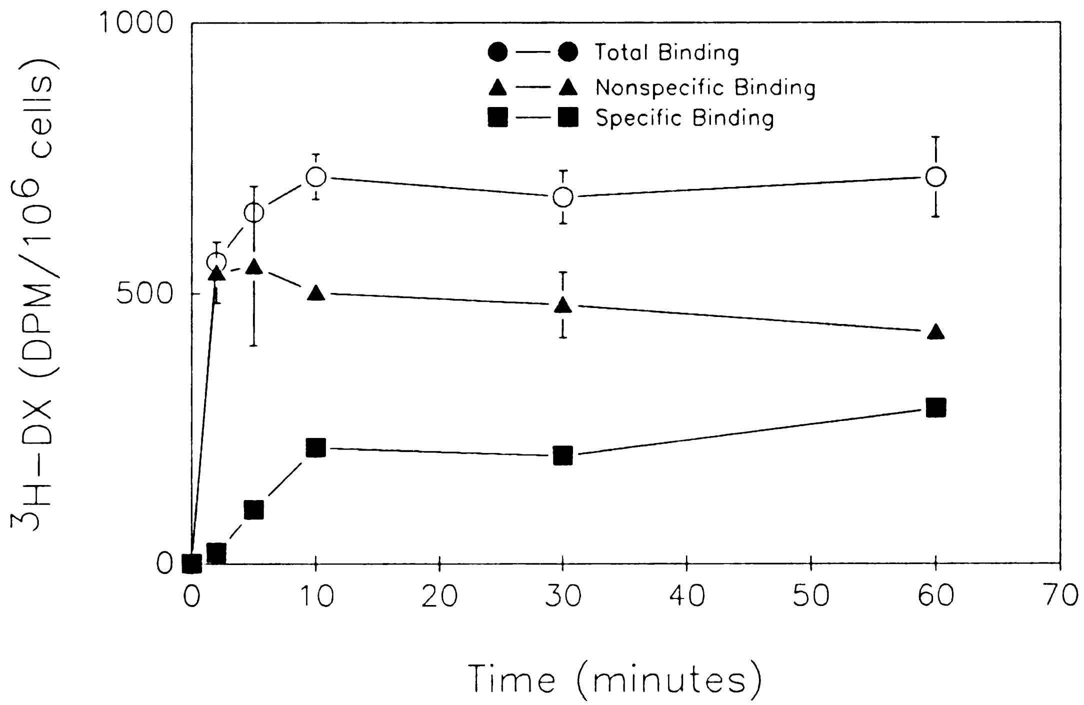
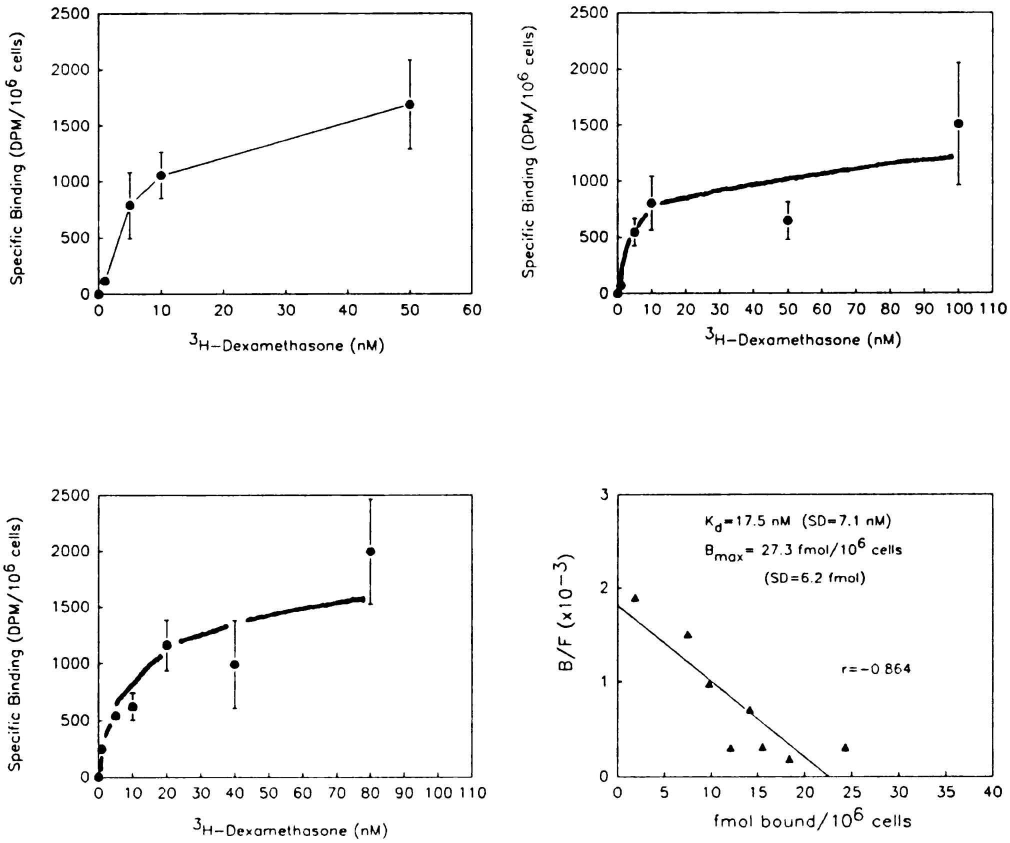

1000
500
0
3H−DX (DPM/10^6 cells)
Total Binding
Nonspecific Binding
Specific Binding
0
10
20
30
40
50
60
70
Time (minutes)

Figure 5.3 Specific binding and Scatchard plots of 70Z/3 cells incubated with ^{3}H-dexamethasone. Cells were incubated for 1 hour with ^{3}H-dexamethasone with or without a 500-fold excess unlabelled DX. Points on specific binding curves represent the mean ± SD of 3 samples. Scatchard analysis was performed by linear regression and represents the combination of all available saturation binding data.


Specific Binding (DPM/10^6 cells)
3H-Dexamethasone (nM)
B/F (x10^-3)
fmol bound/10^6 cells
Kd=17.5 nM (SD=7.1 nM)
Bmax= 27.3 fmol/10^6 cells
(SD=6.2 fmol)
r=-0.864

Chapter 6

Alteration of Murine B-cell Development and Function by Chronic Exposure to Prednisolone: A Role for Apoptosis

SUMMARY

Prednisolone (PD) is commonly used for the treatment of inflammation created by injury or disease (such as arthritis, allergy, and asthma). While it is well documented that pharmacologically used glucocorticoids (GC) cause thymic atrophy due to induction of programmed cell death (apoptosis) in immature T-cells, the effects of PD on normal B-cell development was virtually unknown. Using an *in vitro* murine bone marrow (BM) culture system, it was found that 10^{-8} M, 10^{-7} M, and 10^{-6} M PD caused 36%, 73%, and 85% inhibition of the BM B-cell response to the T-cell independent antigen, trinitrophenol-lipopolysaccharide (TNP-LPS). Additional studies were performed to ascertain the *in vivo* effects of PD. Levels of plasma PD that reached only 2 ng/ml in mice 10 days after implantation of PD pellets were nevertheless sufficient to cause splenic and thymic atrophy and decreased white blood cell counts. In addition, flow cytometric data revealed that there was a 30% decrease in cells of the lymphocyte compartment of the BM and an approximately 60% decrease in proportion of $B220^+$ and sIg^+ cells. Further, there was a 60% reduction in $B220^+sIgM^-$ pre-B cells while immature $sIgM^+sIgD^-$ were completely depleted from the BM of PD-treated mice. The proportion and absolute number of mature $sIgM^+sIgD^+$ cells in the BM was not different between control and PD-treated mice. These changes were accompanied by a 60% reduction in the ability of the BM to respond to TNP-LPS. Flow cytometric cell

cycle analysis of BM cells cultured for 16 hours in the presence of 10^{-7} M PD revealed that approximately 40% of IgM^+ and $B220^+$ cells resided to the left of G_0/G_1 in a region associated with apoptotic cells previously termed the A_0 region. Taken together, these data indicate that low levels of PD significantly altered BM B-cell development by depletion of cells in the B-lineage compartment. Further, this depletion appeared to be caused by PD-induced apoptosis. The data also demonstrate the extreme immunosuppressive potency of PD which significantly altered lymphopoiesis at nanogram levels when administered *in vivo*.

INTRODUCTION

PD is a synthetic GC analog that is frequently used in human (and veterinary) medicine for the treatment of autoimmune diseases such as rheumatoid arthritis and lupus erythematosus, allergy, asthma, various hematopoietic malignanies, etc. (Abrams, 1983; Szefler, 1989) Long-term therapy in which PD (or other GC) are administered systemically for several weeks lead to a plethora of side effects including cataracts, hypertension, gastrointestinal disorders, osteoporosis, psychosis, truncal obesity, increased susceptibility to infection, etc. (Axelrod, 1989; Reynolds, 1989). While the purpose of this dissertation project was to determine the effects of endogenously produced GC at physiological concentrations on B-cell lymphopoiesis, the widespread pharmacological use of PD as an immunosuppressive drug made the questions addressed in this chapter a logical extension of the dissertation project.

Our interest in determining whether PD also altered lymphopoiesis was further heightened by the observation that a single dose of 60-80 mg PD had been shown to cause a transient lymphopenia and monocytopenia in humans (Yu *et al.*, 1974; Fauci, 1976). In addition, alternate-day therapy (5 to 120 mg prednisone) in patients with a variety of illnesses resulted in transient lymphopenia and depressed *in vitro* responses to various mitogens and antigens (Fauci & Dale, 1975). All of this suggested PD altered lymphopoiesis. Yet, there was no indication in the literature about the possible effects of PD on immature and pre-B-cells in the BM. This was a logical question to address given that the absolute number of peripheral blood B-cells was reduced after acute doses of PD and that PD also had been shown to cause programmed cell death (apoptosis) in immature thymocytes (Wyllie, 1980; Telford *et al.*, 1991). Further, McConkey *et al.* (1991) reported that methylprednisolone caused apoptosis in human neoplastic B-cells *in vitro* while there was no apparent effect on normal tonsillar B-cells. Since it was shown in Chapters 2 and 3 of this dissertation that a number of GC types induced apoptosis in B-lineage BM cells, there was reason to suspect PD might have similar effects. Although PD has proven to be a valuable pharmacological agent for a variety of disease states, a more thorough investigation was needed regarding its potential effects on the production of leukocytes especially lymphocytes.

The studies presented here have utilized both *in vitro* and *in vivo* techniques described in previous chapters to investigate the effects of PD on BM B-cell development and function. While it might be expected that these results would parallel those found in previous chapters, there were some intriguing differences which suggest that synthetic GC and endogenously produced GC do not always have the same immunological effects.

It should be noted that some of the data shown in this Chapter were collected by Bryan Voetberg as part of an undergraduate project. It is presented here because he was trained and supervised by Beth Garvy who also collected a considerable part of the data.

METHODS

Materials

Bovine serum albumin (GF-BSA), prednisolone (PD), flumethasone, Histopaque 1083, and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). Prednisolone disodium phosphate (PDSP) was obtained from Steraloids (Wilton, NH). RU 38486 was a gift from Roussel-Uclaf (Romainville, France). Fluorescein isothiocyanate (FITC) conjugated goat anti mouse IgM+G and avidin conjugated FITC were purchased from Tago, Inc. (Burlingame, CA). B220 antibody was collected from the supernatant of the RA3.6B2 cell line (a gift from R.A. Miller, University of Michigan, Ann Arbor, MI) and biotinylated. Solid-phase extraction columns were purchased from Burdick & Johnson Division of Baxter Healthcare Corp. (McGaw Park, IL).

Short Term Bone Marrow Culture

The effects of PD on immature B-cell function was assessed using a short term bone marrow (BM) culture system described in detail in Chapter 4. Briefly, murine BM cells were flushed from femurs and tibias and red blood cells removed. BM cells were suspended at a concentration of 5×10^5 small round nucleated cells per ml culture medium consisting of HEPES-sodium bicarbonate buffered RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 IU penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 5 x 10^{-5} M 2-mercaptoethanol, 0.5% GF-BSA, and 5% FCS. Aliquots of 0.6 ml BM cell suspension were placed in 24 well plates and treated with one or more of the following: 0.01 μg/ml trinitrophenylated lipopolysaccharide (TNP-LPS), PD (disodium phosphate) dissolved in RPMI-1640, or RU 38486 dissolved in ethanol. Additives were diluted into RPMI-1640 and the final concentration of ethanol presented to the cells was never greater than 0.05%. Finally, the cultures were placed in a 37° C humidified incubation chamber under an atmosphere of 10% CO_2, 7% O_2, and 83% N_2.

Plaque Forming Cell Assay for Quantitation of the Response of Bone Marrow B-Cells to TNP-LPS

After 5 days of incubation, a plaque assay as described in Chapter 4 was used to quantitate anti-TNP responsive BM B-cells by their ability to lyse TNP-coupled sheep red blood cells (Medina *et al.*, 1988). Data are expressed as plaque forming cells (PFC) per 10^7 small round nucleated cells originally placed into culture.

Preparation and Implantation of Prednisolone Pellets

Male A/J or CAF_1 mice (The Jackson Laboratories, Bar Harbor, ME) aged 5-7 weeks were surgically implanted (as described in chapter 3) with pellets consisting of 10 mg PD in a 30 mg cholesterol matrix or given 40 mg cholesterol without steroid (sham control). Ten days after pellet implantation, mice were anesthetized and blood was collected by severing the subclavian artery. Blood was drawn within 90 sec of disturbing each cage to minimize the release of endogenous glucocorticoids. Plasma from each mouse was obtained by centrifugation and stored at -20° C until assayed for PD concentration. White blood cell (WBC) counts were obtained using Unipettes (Becton, Dickinson, & Company, Rutheford, NJ) according to the manufacturer's instructions. Bone marrow was flushed from femurs and tibias and RBC removed by centrifugation over Histopaque 1083 (Sigma Chemical Co., St. Louis, MO). After washing, viability was determined by trypan blue exclusion. Part of the BM from each mouse was used for phenotypic analysis and the remaining cells were placed in culture at 10^6 total cells/well, treated with TNP-LPS, incubated, and assayed as previously described.

Assay for Plasma Prednisolone Concentrations

Plasma PD concentrations were determined by Helen Mayer in the Michigan State University Mass Spectroscopy facility. PD was oxidized to 1,4-androstadien-3,11,17-trione using pyridinium chlorochromate followed by analysis using gas chromatography/electron capture negative ionization/mass spectrometry (GC/ECNI/MS). Mouse plasma samples were spiked with 3 ng flumethasone in 10 μl methanol. Steroids were extracted using 200-mg C18 solid-phase extraction columns as previously described (Kayganich *et al.*, 1990). The columns were washed with 4 ml water and the steroid eluted with 4 ml methanol. After evaporation under nitrogen, extracts were oxidized using pyridinium chlorochromate for 6 hours (Watson & Kayganich, 1989). To remove oxidation reagents, samples were placed on silica solid-phase extraction colums, washed with methylene chloride, and eluted with ethyl acetate. After evaporation under nitrogen, the residue was reconstituted in 75 μl of ethyl acetate with 4-6 μl required for GC/ECNI/MS analyses. A standard curve was prepared using normal mouse plasma spiked with 3 ng flumethasone plus 0, 1, 5, 10, or 20 ng of PD, and the samples processed as described. Methane ECNI mass spectral data were obtained for each sample on a JEOL JMS-AX505H mass spectrometer as described elsewhere (H. Mayer, submitted). A J&W 15 m x 0.25mm id x 0.25 μm DB-1701 column (Folsom, CA) was directly inserted in the mass spectrometer source.

Phenotypic Labelling of Bone Marrow Cells for Flow Cytometric Analysis

Aliquots of 10^6 BM cells were incubated with 2.5 μg FITC-conjugated goat anti-mouse IgM+G antibody (Tago, Burlingame, CA), 2 μg FITC-conjugated sheep anti-mouse IgD (The Binding Site, Birmingham, England), 3 μg/ml phycoerythrin-conjugated goat anti-mouse IgM (Jackson Immunoresearch Labs, West Grove, PA), or 1.5 μg B220 antibody for 30 minutes at 4°C with occasional mixing. Biotinylated B220 labelled cells were incubated as above with streptavidin-conjugated FITC (Vector, Burlingame, CA). After washing with phosphate buffered saline (pH 7.4) plus 5% heat-inactivated FCS and 0.15% sodium azide, cells were suspended in 1 ml and held on ice until analysis.

Detection of Apoptosis and Analysis of Cell Cycle Status of Bone Marrow B-cells Cultured with PD

BM cells were placed into culture with PD as described. The cells were harvested 16 hours later, phenotypically labelled as described above, and fixed in 50% ethanol with PBS plus 50% HIFCS. After washing to remove the fixative, the DNA of these cells was stained with propidium iodide (PI) staining reagent consisting of 50 μg/ml PI and 0.5 mg/ml RNase A in PBS, pH 7.4, for 1 hour at room temperature. The cells were placed on ice for analysis the same day.

To analyze cell cycle status of subsets of B-cells within the bone, cells also were labelled with a fluorochrome-tagged phenotypic B-cell marker and analyzed using an Ortho Cytofluorograph 50H/2150 computer system as described in Chapters 2 and 3. Two-color phenotypic and cell cycle analysis was performed using an 80386 computer system with Acqcyte software (Phoenix Flow Systems, Palo Alto, CA). All analyses were carried out as described previously in Chapters 2 and 3.

Statistical analysis.

The results were analyzed for significant differences using analysis of variance followed by Student Newman-Keuls post-hoc tests, where appropriate, for determining between-group differences. Student's *t-tests* were used for determining between-group differences where only two groups were compared. Differences were considered significant at $p<0.05$.

RESULTS

Inhibition of BM B-cell Function by PD In Vitro

In order to assess the effects of PD on BM B-cell function an *in vitro* system was used which allows for the analysis of a clonally selected population of cells responsive to the T-cell-independent antigen, TNP-LPS. Figure 6.1 shows that concentrations of 10^{-8} M PD and greater caused a significant inhibition of PFC production, with maximal inhibition occurring around 10^{-6} M PD. PFC production was reduced to less than 30% of control by 10^{-7} M and less than 20% by 10^{-6} M PD. A concentration of 10^{-8} M PD caused variable results with some experiments showing slight inhibition of PFC production, while others showed no effect indicating that the minimum effective dose *in vitro* was between 10^{-8} and 10^{-7} M PD.

The GC receptor antagonist, RU 38486, partially protected BM B-cells from PD (Figure 6.2). When used alone, RU 38486 at 10^{-6} M did not affect PFC production; but when added to cultures along with 10^{-7} or 10^{-6} M PD, PFC production was 50% greater than in cultures with PD alone. These data indicate that PD had a significant inhibitory effect on BM B-cell response to TNP-LPS at fairly low concentrations and that the effect was most likely exerted through the classical GC receptor.

Effects of PD on BM B-cells In Vivo: Implantation System

In order to evaluate the effects of PD *in vivo*, mice were surgically implanted with pellets containing either PD or vehicle alone. Plasma PD levels at day 10 post surgery were a modest 2.00 ± 3.16 ng/ml in the PD-treated mice compared to a background of 0.12 ± 0.35 ng/ml in control mice (Table 6.1). Analysis of PD plasma levels at 48 hours after PD pellet implantation revealed a mean of 8.30 ± 0.87 ng/ml in mice with implants containing steroids. These concentrations are very low compared to the peak plasma concentrations found in normal humans which have been reported to be between 160-380 ng/ml for a single oral dose of 10 mg PD (Rees & Lockwood, 1982). At 10 days after implantation, the weights of thymuses from PD-treated mice were 50% lower than those of sham controls. WBC counts also were decreased by 50% in PD treated mice while spleen weights were less than 70% of controls (Table 6.1). These data indicate that very modest levels of plasma PD caused a significant decline in the cellularity of peripheral lymphoid organs consistent with effects reported in humans (Yu *et al.*, 1974; Fauci & Dale, 1975).

Light scatter profiles obtained from flow cytometric analysis in which forward scatter is an indicator of cell size and side scatter indicates cell granularity indicated that there was a modest decrease in the proportion of small, non-granular cells in the BM of PD-treated mice (24.5% lymphocytes) as compared to controls (31.9% lymphocytes)

(Figure 6.3). The decrease in small, nongranular lymphocytes was accompanied by an increase in the proportion of larger, more granular cells (Figure 6.3). Fluorescence analysis of the BM B-lineage subpopulation indicated that 10 days of PD exposure caused a significant decrease in both surface immunoglobulin (sIg) and B220-bearing BM cells. The proportion of cells expressing either sIg or B220 was on average 40% lower in PD-treated mice than in control mice (Figure 6.4) 10 days after implantation. These decreases in the proportion of B-lineage cells also represented real decreases in absolute number of cells since the overall BM cellularity was unchanged by the PD treatment (data not shown). The fluorescence intensity of expression of sIg^+ or $B220^+$ on cells (an indicator of cell surface density of surface molecules) from PD-treated mice was the same as in controls (data not shown). These data indicate that chronic elevation of plasma PD caused a significant decrease in the B-cell compartment of the bone marrow.

Analysis of BM B-lineage cells in PD-treated mice at day 10 using dual fluorescence labelling indicated that the $sIgM^+sIgD^-$ immature B-cells were virtually depleted leaving residual B-cells that were $sIgM^+sIgD^+$ (Figure 6.5). However, the proportion and absolute number of BM $sIgM^+sIgD^+$ cells in the PD-treated mice was not different than in the controls. There was also 3-fold decrease in $B220^+sIgM^-$ pre-B cells in the BM of PD-treated mice. Only a small residual population remained (Figure 6.5), and it is not known whether or not these residual pre-B cells represented large, cycling or small, quiescent cells.

To determine if residual BM B-cells from PD-treated mice were functional, cells were placed into culture and challenged with TNP-LPS for five days as previously described. BM from PD-treated mice produced only about 40% as many anti-TNP PFC relative to control mice (Table 6.2). In spite of the fact that BM cells were no longer exposed to PD during this 5 day culture period, reduced numbers of plaques were obtained. This suggested that the BM cells which normally responded to TNP-LPS had either been depleted or were nonresponsive during the culture period.

Induction of Apoptosis by Prednisolone In Vitro

Since PD has been shown to induce apoptosis in thymocytes (Wyllie, 1980; Telford *et al.*, 1991) and the previous chapters of this dissertation indicated that GC induced apoptosis in BM B-lineage cells, a flow cytometric assay was used to determine if PD also would induce apoptosis in BM B-lineage cells. Using the method described in Chapter 2 of this dissertation, BM cultured for 16 hours with PD was subjected to two-color cell cycle analysis. $B220^+$ and IgM^+ cells were examined for light scatter characteristics and DNA staining patterns using PI. Both $B220^+$ and IgM^+ cells underwent a downward shift when examined for forward versus side scatter (Figure 6.6). This shift is characteristic of a decrease in cell size and an increase in cell density which is commonly described in apoptotic thymocytes (Telford *et al.*, 1991; Wyllie & Morris, 1982) and was observed in DX-treated BM B-cells (see Figure 2.2).

Cell cycle analysis of B220- or IgM-gated B-lineage cells revealed the formation of a discrete peak to the left of G_0/G_1 in the "hypodiploid" area which has been previously termed the A_0 region (Telford *et al.*, 1991). Treatment of cultures with 10^{-7} M PD for 16 hours caused an accumulation of 40.0% of $B220^+$ and 42.9% of IgM^+ cells in the A_0 region of the cell cycle (Figure 6.7). Cell culture alone caused an accumulation of 12.2% and 18.8% $B220^+$ and IgM^+ cells, respectively, in the A_0 region compared to just over 1% in the A_0 region of freshly isolated $B220^+$ and IgM^+ cells. Simultaneous addition of the GC receptor antagonist RU 38486 and PD reduced the size of the A_0 region to near background levels (Figure 6.7). Zinc (500 μM), a commonly cited inhibitor of apoptosis, also reduced accumulation of events in the A_0 peak to background levels when added to cultures (data not shown). These data indicate that PD induced apoptosis in BM B-lineage lymphocytes *in vitro* as was previously reported for thymocytes and that this induction was a mediated by GC receptors.

DISCUSSION

The data presented here showed that PD had a significant suppressive effect on both the function and development of BM B-lineage cells. These were significant findings given that PD is widely used as a pharmacological agent. PD significantly inhibited the *in vitro* BM response to the T-cell independent antigen, TNP-LPS, at concentrations which corresponded to those found in normal human plasma after a single oral dose of 10 to 60 mg ($4x10^{-7}$ to $3x10^{-6}$ M) (Rees & Lockwood, 1982). This inhibition appeared to be mediated by the classical GC receptor since RU 38486, a known GC receptor antagonist (Moguilewsky & Philibert, 1984), provided some protection from the effects of PD. It is probable that this decreased response to TNP-LPS *in vitro* was due to PD induction of apoptosis in BM B-lineage cells since subsequently a significant number of cells cultured with PD were induced to undergo apoptosis after only 16 hours of exposure. Though activated cells have been shown to be resistant to induction of apoptosis (Liu *et al.*, 1989), in this case PD was added to cultures at the same time as TNP-LPS and may have induced the start of the death pathway before the cells could be fully activated. This would be consistent with reports in the literature and in Chapter 4 of this dissertation which indicated that GC inhibited early events in B-cell activation but had no effect on cells when added to culture 48 hours or more after stimulation (Bowen & Fauci, 1984; Roess *et al.*, 1983; Luster *et al.*, 1988).

A unique delivery system was used to evaluate the effects of chronic *in vivo* exposure to PD on BM B-cell development. The plasma PD levels found 10 days after PD pellet implantation were very low (2 ng/ml) compared to those reported for a single pharmacological dose (160-1200 ng/ml) (Rees & Lockwood, 1982; Green *et al.*, 1978). Though these concentrations were very low, PD treated mice had significant lymphopenia in peripheral tissues. Thymus and spleen weights and white blood cell counts were decreased by as much as 50%; however, consistent with other reports (Fauci, 1975c; Ku & Witte, 1986; Sabbele *et al.*, 1987), BM cellularity was unchanged.

Contrary to the longstanding belief that administration of pharmacological doses of GC causes redistribution of peripheral lymphocytes to the BM (Fauci, 1975c; Cohen, 1972), these data indicated that B-lineage cells were depleted from the BM of mice chronically exposed to modest levels of PD. Immature $sIgM^+sIgD^-$ B-cells were completely depleted from the BM of PD-treated mice. A small population (about 2%) of mature $sIgM^+sIgD^+$ B-cells remained in the BM; however, the proportion and absolute numbers of these cells were not different than in the BM of control mice indicating that mature B cells had not been redistributed to the BM as suggested in the literature (Fauci, 1975c). The decrease in the number of immature B-cells that are thought also to be responsive to antigen undoubtedly contributed to the significant reduction in anti-TNP plaque-forming cell production found in the BM of PD-treated mice.

Interestingly, a small subpopulation of B220$^+$sIgM$^-$ pre-B cells were found in the BM of PD-treated mice. It was not possible to determine from these data if these pre-B cells were large cycling cells, small quiescent cells, or a combination of the two. An appealing hypothesis is that they were large pre-B cells that were arrested in G_1 of the cell cycle, a phenomenon which has been demonstrated in a transformed lymhocyte cell line (Harmon *et al.*, 1979). Alternatively, these pre-B cells may have represented a wave of regenerating cells which were progressing through the stages of B-cell lymphopoiesis to replenish the depleted BM. If true, it would indicate that an early precursor (probably earlier than TdT$^+$ cells, (Vines *et al.*, 1980; Hayashi *et al.*, 1984) was not adversely affected by PD.

Regardless, these findings were significantly different from those reported in Chapter 3. Unlike the BM of-PD treated mice, the BM of CS-treated mice was completely depleted of B220$^+$sIgM$^-$ pre-B cells. Further, an increased number of mature sIgM$^+$sIgD$^+$ B-cells were found in the BM of CS-treated mice while in PD-treated BM the number of mature B-cells was unchanged. The differences between the two GC may have been a dose effect. Although PD has been reported to have a high potency compared to natural GC (Bach & Strom, 1985), plasma PD concentrations were only around 6 nM at day 10, while the molar concentrations of plasma CS were about 150 times greater (about 1 μM).

Finally, it was shown that PD also induced apoptosis in B220$^+$ and sIgM$^+$ BM B-cells *in vitro*. The concentration of PD used (10^{-7} M) corresponded to those used to inhibit anti-TNP PFC production and was much higher than plasma PD levels in pellet-implanted mice. Though indirect, these data indicated that PD-induced apoptosis could have been responsible for the depletion of B-lineage cells in the BM of PD pellet-implanted mice as was shown for CS-treated mice in Chapter 3 of this dissertation. Together the data clearly indicate that, contrary to early published reports (Fauci, 1975c; Cohen, 1972; Fauci, 1976), very low concentrations of PD and moderate concentrations of GC have adverse effects on BM B-cell development. These findings may have important implications in the strategies used for prescribing predisolone and other pharmacologically used glucocorticoids.

Table 6.1 Plasma prednisolone concentration, thymus and spleen weights, and white blood cell counts of mice 10 days after pellet implantation.

	Control	PD Treated
Plasma PD (ng/ml)	0.12±0.35	2.00±3.16
Thymus Weight (mg)	23±6	11±4*
Spleen Weight (mg)	54±8	36±5*
WBC/ml ($x10^{-6}$)	4.1±1.2	2.1±0.8*

Data is presented as the mean ± SD of 8 mice per group. PD, prednisolone.

*Significantly ($p<0.05$) different from controls which received no steroid.

Table 6.2 Plaque forming cell response to TNP-LPS of bone marrow cells from sham control or prednisolone-treated mice 10 days after pellet implantation.

Treatment	PFC/10^7 BM cells
Sham Control	13,199 ± 4851
Prednisolone	5,358 ± 3605*

*significantly different from controls, $p<0.05$

Figure 6.1 Plaque forming cell response of bone marrow B-cells to TNP-LPS in the presence of various concentrations of prednisolone. Data points represent the mean (±SD) of quadruplicate cultures. Data are representative of 4 separate experiments. Asterisks indicate significant differences ($p<0.05$) from cultures without PD.

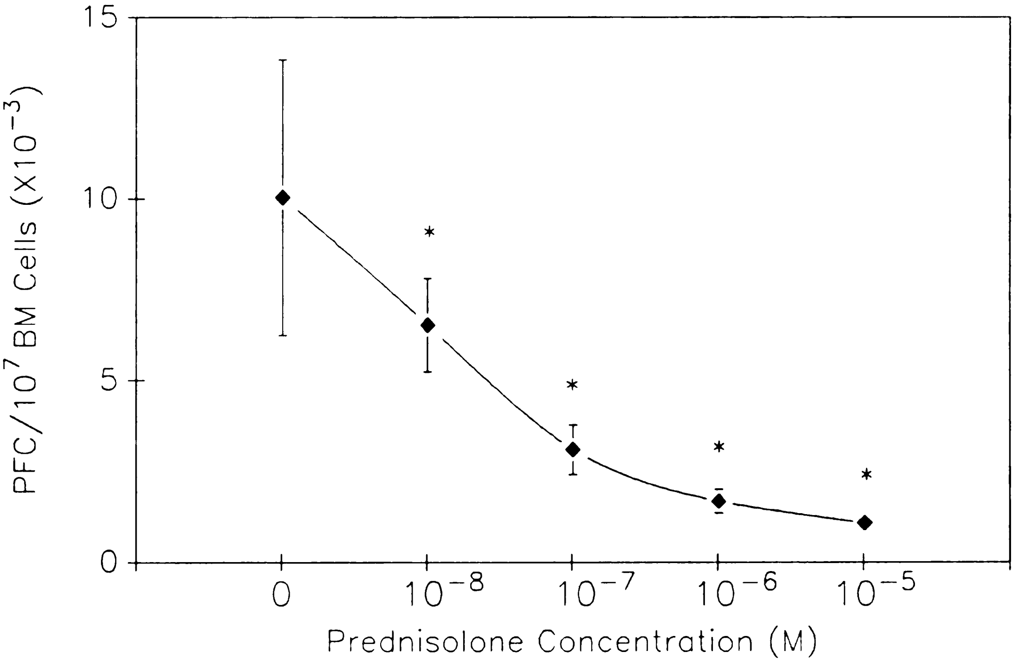

15
10
5
0
PFC/10^7 BM Cells (X10^-3)
0
10^-8
10^-7
10^-6
10^-5
Prednisolone Concentration (M)
*
*
*
*

Figure 6.2 Plaque-forming cell response of bone marrow B-cells to TNP-LPS in the presence of various concentrations of prednisolone with or without 10^{-6} M RU 38486. Data are expressed as the mean (±SD) of quadruplicate cultures and are representative of 4 separate experiments. Asterisks indicate those cultures with PD that were significantly different ($p<0.05$) from cultures with PD and RU 38486.

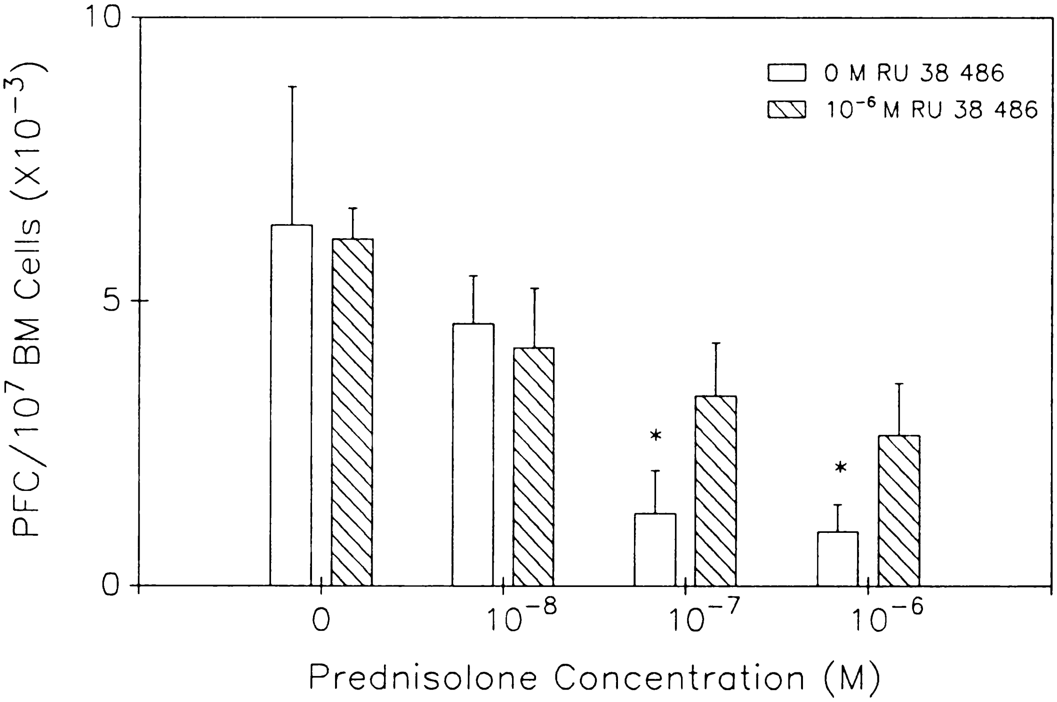
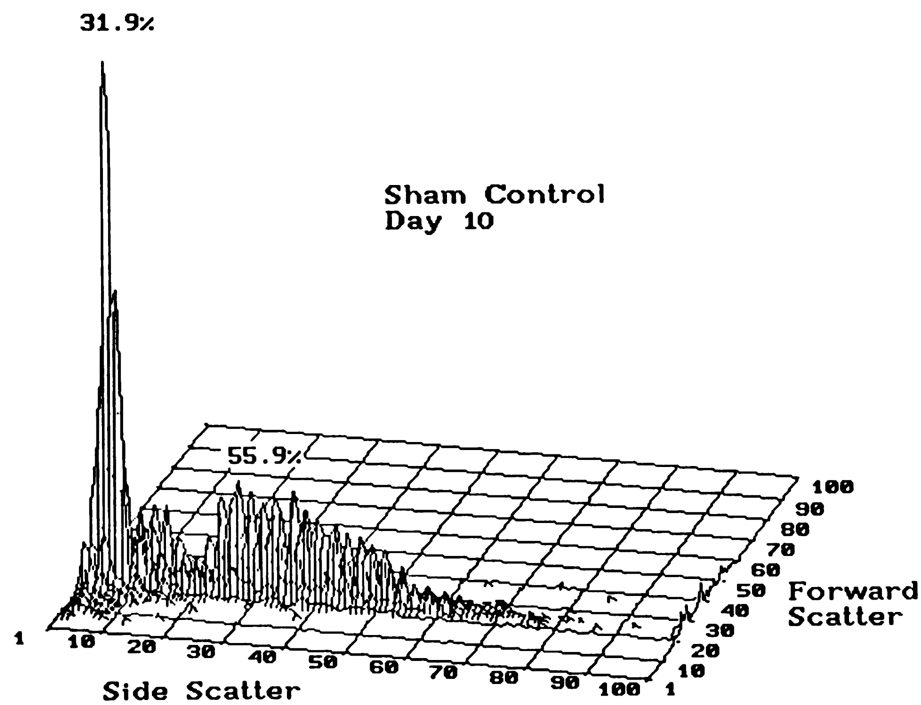

10
5
0
PFC/10^7 BM Cells (X10^-3)
0 M RU 38 486
10^-6 M RU 38 486
*
*
0
10^-8
10^-7
10^-6
Prednisolone Concentration (M)

Figure 6.3 Light scatter profiles of bone marrow from sham control and prednisolone-treated mice 10 days after pellet implantation. Whole BM was examined for light scatter characteristics using flow cytometry. Forward scatter is a measure of cell size and side scatter is an indicator of cell internal characteristics (granularity). The proportion of small, nongranular and large, granular cells are indicated. Each cytogram represents an accumulation of 10,000 events (or cells) and is representative of 8 mice in a single experiment and 3 separate experiments.


31.9%
Sham Control
Day 10
55.9%
Forward Scatter
Side Scatter
1 10 20 30 40 50 60 70 80 90 100
1 10 20 30 40 50 60 70 80 90 100

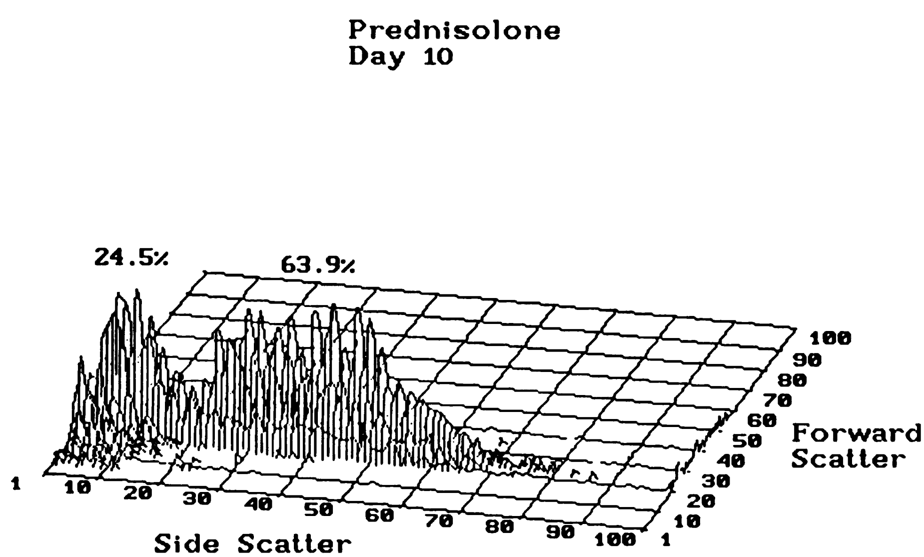

Prednisolone
Day 10
24.5%
63.9%
Forward Scatter
Side Scatter
1 10 20 30 40 50 60 70 80 90 100
1 10 20 30 40 50 60 70 80 90 100

Figure 6.4 Proportion of sIg^{+} and $B220^{+}$ cells in the bone marrow of mice 10 days after treatment with prednisolone-containing pellet implants. One-color flow cytometry was used to determine the proportion of sIg^{+} and $B220^{+}$ cells in the BM of sham-control and PD-treated mice. Data are expressed as the mean ± SD of 8 mice and are representative of 2 separate experiments. Asterisks indicate significant ($p<0.05$) differences from sham controls.

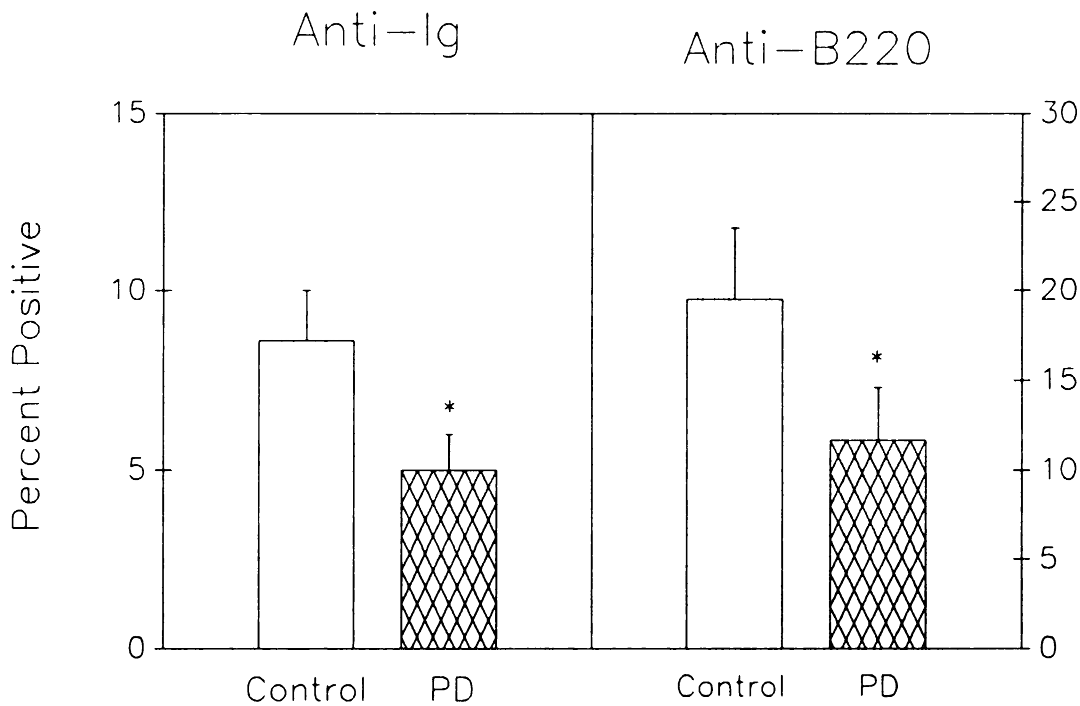

Anti-Ig
Anti-B220
Percent Positive
15
10
5
0
30
25
20
15
10
5
0
*
*
Control
PD
Control
PD

Figure 6.5 Two-color cytograms from the bone marrow of sham-control and prednisolone-treated mice 10 days after pellet implantation. BM was labelled with either anti-B220 and anti-IgM or anti-IgD and anti-IgM and analyzed using flow cytometry. Data in the left two panels were plotted as B220 on the x-axis verus IgM on the y-axis. Data in the right two panels were plotted as IgD on the x-axis versus IgM on the y-axis. Region 1 represents $sIgM^{+}sIgD^{-}$ cells in the two right panels. There are no cells in region 1 in the two left panels since all $sIgM^{+}$ cells also express B220. Region 2 represents cells which expressed both IgM and B220 (left panels) or IgM and IgD (right panels). Region 3 contained cells which were unlabelled. Region 4 in the left two panels contained cells which expressed only B220. There were no cells in region 4 in the right two panels since IgD^{+} cells also express IgM. Data are from a single sham-control or prednisolone-treated mouse and are representative of 6 mice.

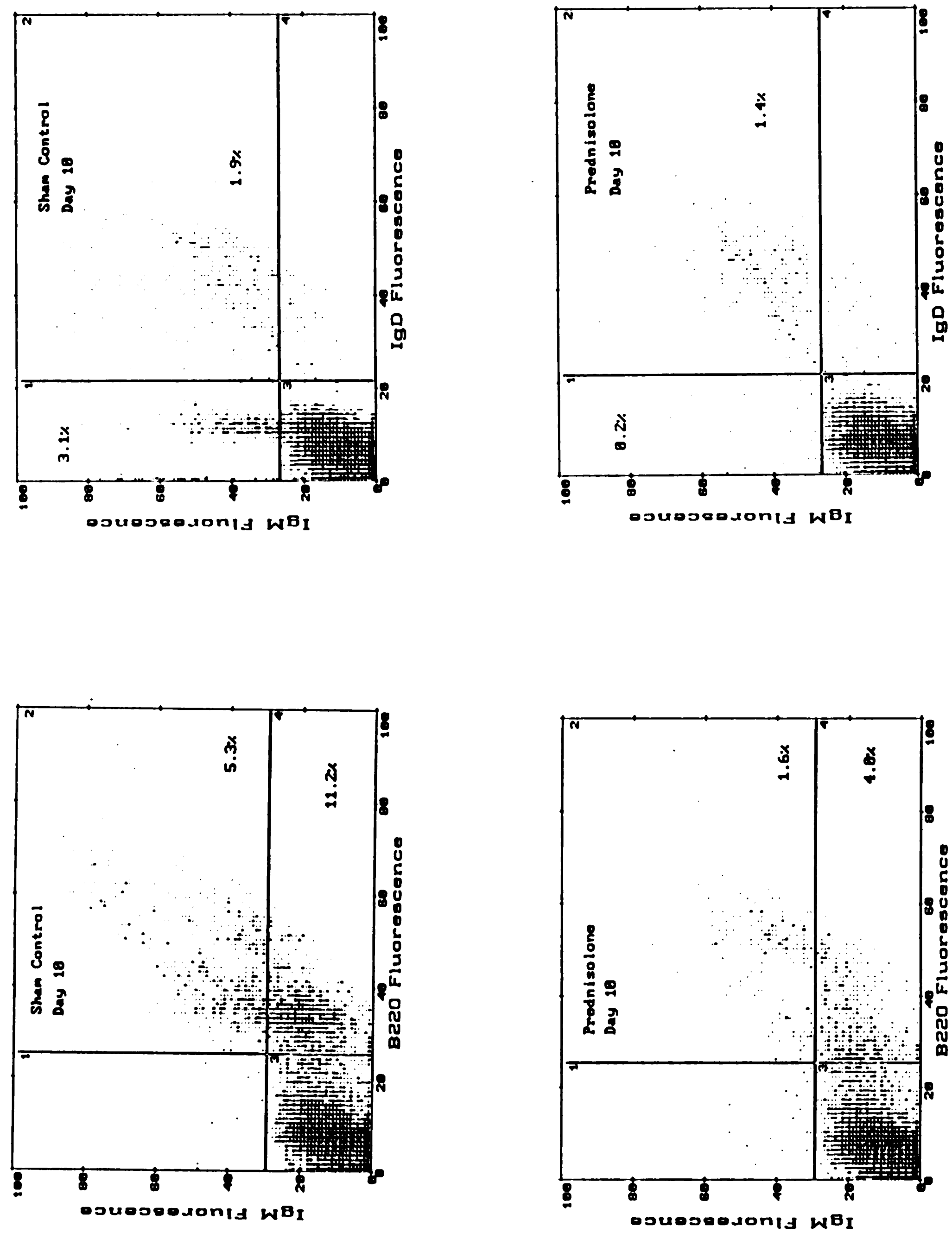
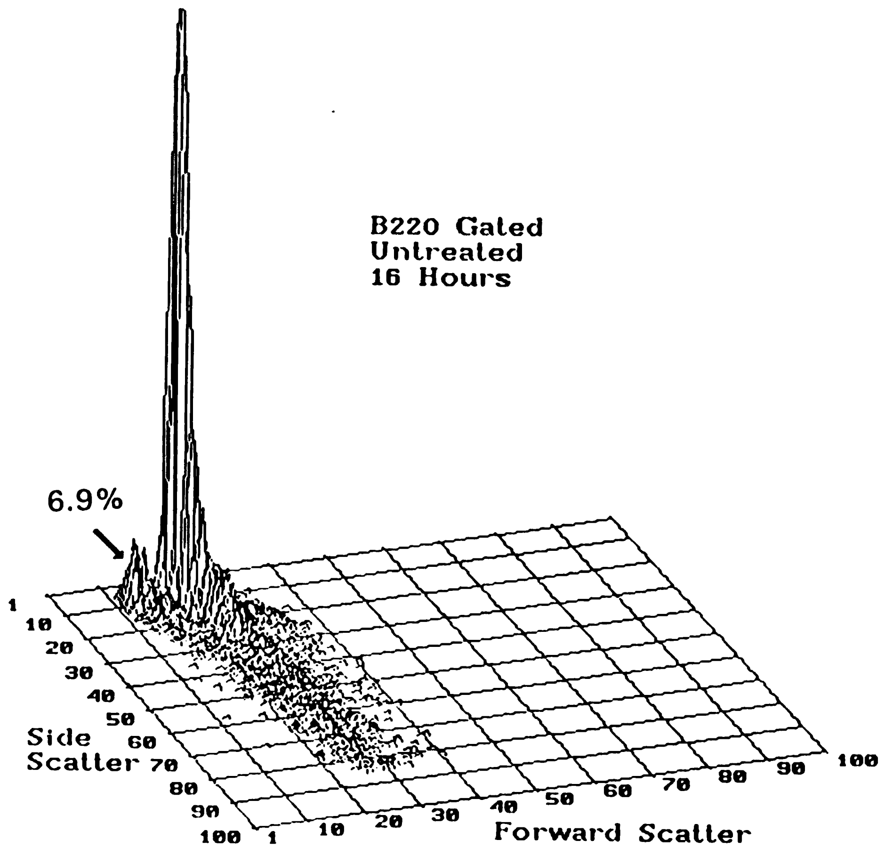

Sham Control
Day 10
1.9%
3.1%
IgD Fluorescence
IgM Fluorescence
Prednisolone
Day 10
1.4%
0.2%
IgD Fluorescence
IgM Fluorescence
Sham Control
Day 10
5.3%
11.2%
B220 Fluorescence
IgM Fluorescence
Prednisolone
Day 10
1.6%
4.0%
B220 Fluorescence
IgM Fluorescence

Figure 6.6 Flow cytometric light scatter profiles of $B220^+$ bone marrow cells cultured for 16 hours with or without 0.1 μM prednisolone. BM was gated for $B220^+$ cells and examined for light scatter characteristics. Forward scatter on the y-axis is indicative of cell size and side scatter on the x-axis is indicative of the granular contents of the cell. Data are representative of 2 separate experiments.


B220 Gated
Untreated
16 Hours
6.9%
Side Scatter
Forward Scatter
1
10
20
30
40
50
60
70
80
90
100

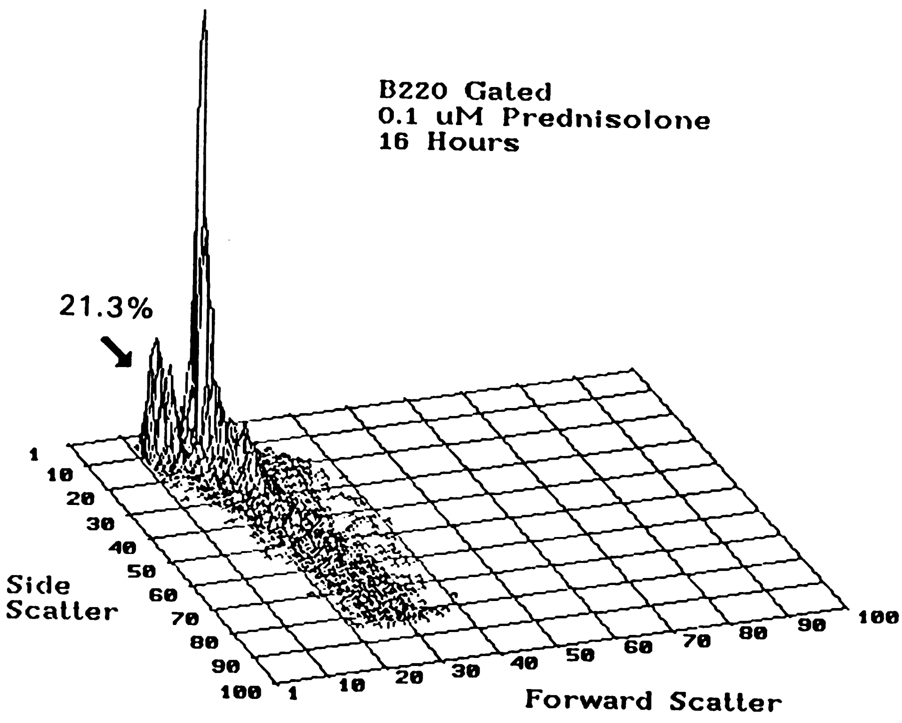
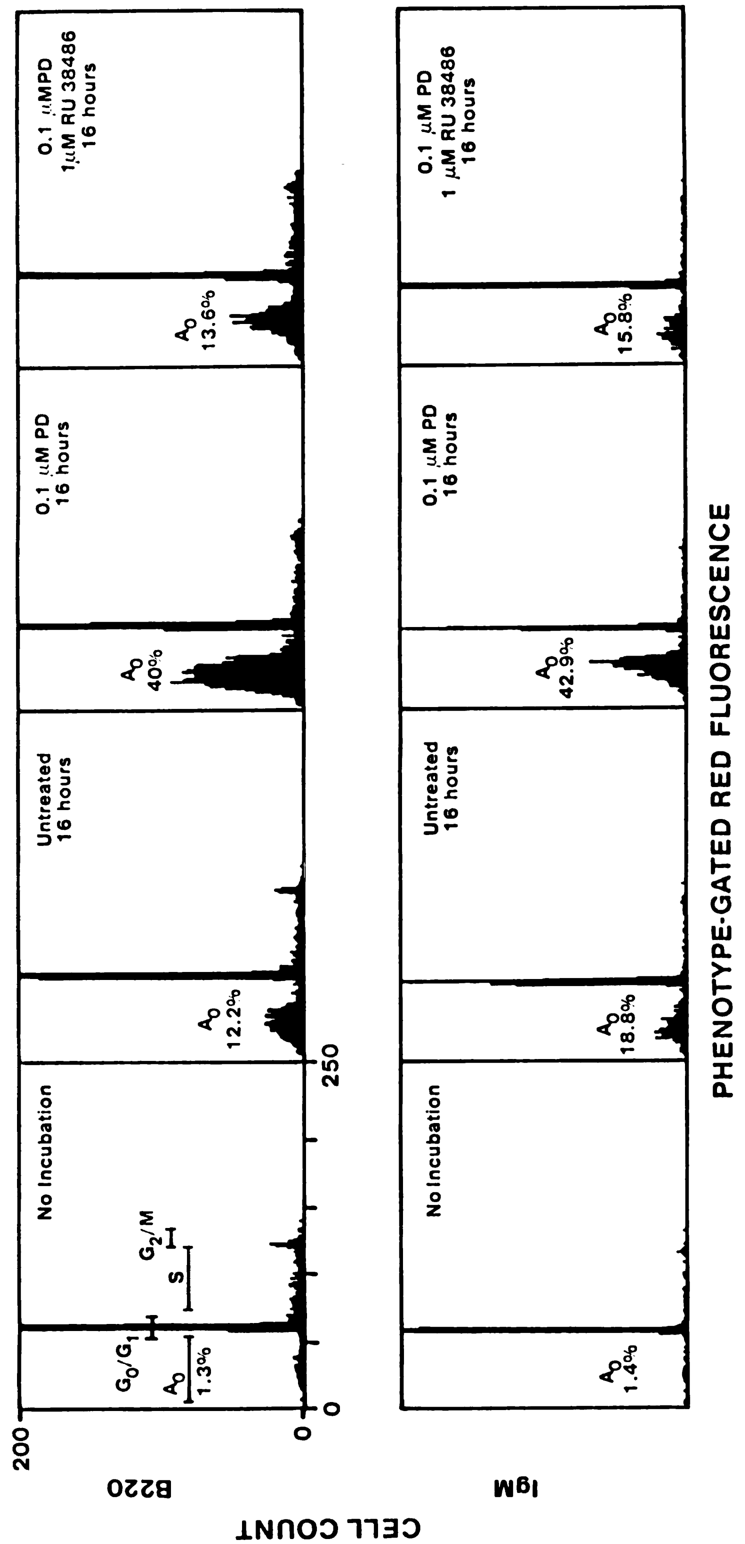

B220 Gated
0.1 uM Prednisolone
16 Hours
21.3%
Side Scatter
Forward Scatter
1
10
20
30
40
50
60
70
80
90
100

Figure 6.7 Cell cycle histograms of bone marrow B220$^+$ and IgM$^+$ cells after 16 hours of culture in the presence or absence of prednisolone with or without RU 38486. BM cells were incubated with the indicated additives and fluorescently labelled with anti-IgM or anti-B220. After fixation, cells were labelled with propidium iodide and analyzed using flow cytometry. Cells were gated to exclude debris and nonspecifically binding antibody. DNA (red) fluorescence is shown on the x-axis and cell count is shown on the y-axis. Phases of the cell cycle are shown.


No Incubation
Untreated 16 hours
0.1 µM PD 16 hours
0.1 µMPD 1µM RU 38486 16 hours
0.1 µM PD 1 µM RU 38486 16 hours
G_0/G_1
S
G_2/M
A_0 1.3%
A_0 12.2%
A_0 40%
A_0 13.6%
A_0 1.4%
A_0 18.8%
A_0 42.9%
A_0 15.8%
200
0
250
B220
IgM
CELL COUNT
PHENOTYPE-GATED RED FLUORESCENCE

Chapter 7

Conclusions and Recommendations

SUMMARY AND CONCLUSIONS

The data presented in this dissertation demonstrated for the first time that concentrations of glucocorticoids (GC) associated with physiological stresses had adverse affects on murine B-cell lymphopoiesis. Normal B-lineage bone marrow (BM) cells were shown to be induced to undergo apoptosis after short-term exposure *in vitro* to physiological concentrations of glucocorticoids. It was also shown for the first time that two-color flow cytometry could be used to quantitate the proportion of a subpopulation of cells undergoing apoptosis in a heterogeneous tissue. Phenotype-gated cell cycle analysis revealed that an accumulation of BM B220$^+$, sIgM$^+$, cells appeared in the A_0 region after only three hours exposure to 1 μM dexamethasone (DX) and increased linearly through 36 hours until greater than 80% B-lineage cells were apoptotic. Surface IgD$^+$ cells were somewhat more resistant to DX-induced apoptosis *in vitro*; but, after a lag of about 12 hours, nearly all sIgD$^+$ cells accumulated in the A_0 region. GC-induced apoptosis was dose dependent and mediated through the classical GC receptor since the receptor antagonist, RU 38486, abrogated the GC affect. These data indicated that like immature thymocytes, immature B-lineage cells could be induced by glucocorticoids to undergo apoptosis and suggested that apoptosis may be an important mechanism in regulating B-cell development. Further, the two-color flow cytometric methodology developed for this project will greatly expand the tissues and cell population that can be evaluated for apoptosis by other investigators.

A unique pellet implantation system also was developed which produced chronically elevated plasma corticosterone (CS) levels in mice at concentrations analogous to those observed during stresses such as malnutrition, thermal injury, trauma, etc. Examination of bone marrow B-lineage cells by two-color flow cytometry indicated that pre-B ($B220^{+}sIgM^{-}$) and immature ($sIgM^{+}sIgD^{-}$) B-cells were completely depleted by day 5 after implantation. However, the proportion of mature $B220^{Bright}sIgM^{+}sIgD^{Bright}$ B-cells increased nearly 2-fold after CS-treatment. These cells undoubtedly were responsible for the normal response to TNP-LPS reported for the BM of CS-treated mice.

Two-color cell cycle analysis indicated that, as early as 6 hours after pellet implantation, a small but significant proportion of $B220^{+}$ and $sIgM^{+}$ cells were in the A_0 region. Further, the appearance of $B220^{+}$ cells in the A_0 region from 12-24 hours after pellet implantation corresponded to a linear decrease in the proportion of BM $B220^{+}$ B-cells observed over the same time period. Since the proportion of $B220^{+}$ cells in the A_0 region was small, the rapid disappearance of $B220^{+}$ cells may have been due to rapid phagocytosis of apoptotic cells (Duvall *et al.*, 1985). Interestingly, though $sIgM^{+}$ cells also were undergoing apoptosis, the proportion of these cells in the BM did not decrease. However, further analysis at 24 hours indicated that $sIgM^{+}sIgD^{-}$ immature B-cells in the BM of CS-treated mice had been replaced by $sIgM^{+}sIgD^{+}$ cells. Cell cycle analysis at 24 hours and at 5 days post-CS implantation revealed that CS treatment had caused a depletion in actively cycling pre-B cells, effectively shutting down B-cell lymphopoiesis. Together these data suggest that the lymphopenia associated with chronic stress may be due to GC-induced depletion of BM pre-B and immature B-cells by apoptosis. Given that the rapid turnover of lymphocytes is costly in terms of energy and nutrients required, it is tempting to speculate that GC may suppress lymphopoiesis in favor of preserving energy for vital organs such as the brain and heart during times of stress.

An *in vitro* short term bone marrow culture system was used to investigate the effects of GC on the ability of BM B-cells to respond to an actual antigenic challenge using the T-cell independent antigen, TNP-LPS. Not surprisingly, physiological concentrations of glucocorticoids reduced the anti-TNP PFC response by as much as 80% compared to control cultures. This inhibition by GC was dose-dependent and reversed by RU 38486. GC were suppressive for anti-TNP PFC production only when added to cultures within 48 hours after stimulation. Factor-rich conditioned medium was unable to overcome the inhibition caused by DX during this time period. If added at 72 hours after antigen, DX had no adverse effects on the capacity of B-cells to respond. Flow cytometric analysis indicated that cultures treated with GC contained only about 2% B-cells while control cultures contained almost 30% B-cells. These data provided evidence that GC had a direct effect on immature antigen-responsive B-cells and probably induced apoptosis before the cells became activated. Once activated, however, GC had no effect on the cultures. This observation is consistent with the notion that activated cells are resistant to apoptosis (Liu *et al.*, 1989).

Though there were similarities in the overall pattern of effects of GC on pre-B and immature B-cells observed *in vivo* and *in vitro,* there were nevertheless some significant differences in outcome which suggest caution towards assumption that observations obtained *in vitro* will be mimicked *in vivo*. For example, whereas the more mature $IgM^{+}IgD^{+}$ cells appeared to be more resistant to the effects of GC administered *in vitro*, they nevertheless eventually succumbed and began entering the apoptotic state. Conversely, $IgM^{+}IgD^{+}$ cells not only survived *in vivo* exposure to analogous levels of GC, but they increased 2-fold and remained functional. The continued availability of stromal cells, macrophages, and growth factors that may have been available *in vivo* but limited *in vitro* may have enhanced or even promoted their survival. Most likely, the intact microenvironment contributed to the difference in the small proportion of B-lineage cells seen undergoing apoptosis *in vivo* compared to the large proportion seen *in vitro*. It is highly probable that phagocytic cells cleared apoptotic B-lineage cells *in vivo* (Duvall *et al*. 1985) while culture compromised the phagocytic capabilities of the BM leading to accumulation of a large number of apoptotic cells.

The *in vitro* data presented in Chapters 2 and 4 of this dissertation provided indirect evidence that bone marrow B-lineage cells have functional GC receptors because a receptor antagonist, RU 38486, was able to abrogate GC-induced effects. However, since it was not known whether murine bone marrow B-lineage cells possessed glucocorticoid receptors, a more direct approach was taken. $B220^+$ B-lineage cells were isolated from murine BM and subjected to a whole-cell binding assay using a tritiated ligand. Since it was not possible to obtain large numbers of B-cells from the BM, radioligand binding was compared to an equivalent number of thymocytes, whose receptor number is established in the literature. The specific binding of ^{3}H-dexamethasone was similar for both thymocytes and $B220^+$ BM cells indicating that B-lineage cells in fact do have several thousand GcR receptors. These data provided additional evidence that GC are able to directly affect BM B-lineage cells. Though the presence of GcR in B-lineage cells suggests that GC can directly alter their development and function, it does not rule out the possibility that GC might also affect B-cell lymphopoiesis by altering the status of essential supporting cells and growth factors in the microenvironemt.

Finally, the various methods used in Chapters 2 through 4 for the analysis of the effects of natural GC on lymphopoiesis were employed to determine the effects of prednisolone (PD), one of the most commonly used pharmacological steroids, on B-cell development. PD significantly inhibited anti-TNP PFC production *in vitro* as was the case of DX and the natural GC. PD also induced apoptosis in BM B-lineage cells *in vitro*. Administration of PD to mice using pellet implants created plasma levels of PD of only a few ng/ml. This, nevertheless, was sufficient to cause a depletion of immature $sIgM^{+}sIgD^{-}$ bone marrow B-cells and reduced $B220^{+}sIgM^{-}$ pre-B cells 3-fold. There was no change in the proportion of $sIgM^{+}sIgD^{+}$ cells in the BM of PD-treated mice. There were some interesting differences between the BM of CS-treated mice and that of PD-treated mice. A small population of pre-B cells was found in the BM of PD-treated mice at day 10 while the BM of CS-implanted mice was completely devoid of pre-B cells at day 5. It may be that these pre-B cells represented regeneration of B-lineage cells after PD had depleted them. This could be determined by examining earlier time points after pellet implantation. Another possibility is that these cells represented pre-B cells which had been arrested in the G_1 phase of the cell cycle but had not undergone apoptosis. Cell cycle analysis of these cells would resolve that issue. Another dissimilarity between PD- and CS-treated mice was the nature of the residual

$sIgM^+sIgD^+$ cells in the BM. While these cells doubled in CS-treated mice, they did not change in PD-treated mice. Also, there was no change in the fluorescence intensity of IgD in the PD-treated mice as opposed to the CS-treated population which expressed bright IgD fluorescence intensity. These differences were reflected in the functional capacity of the BM in response to TNP-LPS. While BM from CS-treated mice responded as well or better than that from controls, the response was significantly inhibited in PD-treated BM. The reasons for these differences were not immediately clear but may have to do with the differences in potencies between the two GC. PD was shown to be much more potent *in vivo* since like CS, it caused a significant impairment in lymphopoiesis at plasma concentrations which were nearly 200-fold less than plasma levels of CS.

RECOMMENDATIONS

The results presented in this dissertation firmly established that physiological concentrations of GC down regulate lymphopoiesis. This is significant evidence that GC are responsible for the lymphopenia observed during chronic stress. It would be a logical extension of these studies to examine the BM of chronically stressed mice for similar changes in lymphopoiesis. Preliminary studies done in this laboratory several years ago indicated that zinc deficiency caused a reduction in the proportion of $B220^+$ and IgM^+ cells of the BM. It is now possible to use two-color flow cytometry to more accurately define those populations and assess the cell cycle state of the cells which were affected by chronic stresses such as zinc deficiency. It will be more difficult to document the presence of apoptotic B-lineage cells in the BM of chronically stressed mice since the apoptotic cells are cleared rapidly. Inhibiting phagocytosis may be an alternative to this problem. Park and Osmond (1991) have used silica *in vivo* to suppress macrophage function, and this method may prove useful in allowing the accumulation of maximal quantities of apoptotic BM cells *in vivo*.

In vitro studies reported in Chapter 2 of this dissertation indicated that while B-lineage cells made up a significant proportion of BM cells undergoing apoptosis, there were other cells of an undefined lineage which also were undergoing apoptosis. The two-color cell cycle analysis used here to quantitate apoptosis in B-lineage cells could easily be used to identify the cells of lineages that were induced to undergo apoptosis by GC. It would also be interesting to know if these cells were important to the microenvironment essential for B-cell lymphopoiesis.

Recently it was discovered that the IL-7 receptor gene has two putative GC response elements upstream of the promoter (Pleiman *et al.*, 1991). IL-7 is a stromal cell secreted cytokine which appears to be an integral part of the regulation of B-cell development (Ucken *et al.*, 1991; Lee *et* *al.*, 1989). If GC are able to down regulate the expression of IL-7 receptors, this might be a singular event that leads to the death of pre-B cells. It would of great interest to know, therefore, what effect GC have on IL-7 receptor expression in BM B-lineage cells. Antibodies to the IL-7 receptor are now available commercially, and IL-7 receptor expression could be assessed using flow cytometry.

Glucocorticoids have also been shown to cause the down regulation of *c-myc* expression in transformed T-cell lines (Yuh & Thompson, 1989; Forsthoefel & Thompson, 1987; Eastman-Reks & Vedeckis, 1986). Though no function has been found for *c-myc*, it is a proliferation associated protoconcogene whose gene product is thought to be important in controlling proliferation. GC-induced down-regulation of *c-myc* has been associated with accumulation of cells in G_1 of the cell cycle (Yuh & Thompson, 1989) in transformed cell lines. It would be interesting to know if GC also down-regulate *c-myc* expression in BM B-lineage cells induced to undergo apoptosis.

Finally, significant attention has been paid recently to an oncogene referred to as *bcl-2* whose overexpression has been reported to inhibit apoptosis in IL-3 dependent B-cell lines deprived of the growth factor (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990). The *bcl-2* gene product has recently been shown to be localized in the inner mitochondrial membrane. Transgenic mice overexpressing *bcl-2* in the thymic cortex have recently been shown to be resistant to GC-induced atrophy *in* *vivo* (Gabriel Nunez, personal communication). Though there is no known function for *bcl-2*, it would be of interest to known if overexpression in BM B-lineage cells would also protect them from the effects of GC. Further, it would be interesting to know what role normal levels of *bcl-2* might play in the apoptotic pathway and its expression level in cells which are both sensitive and resistant to GC-induced apoptosis.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abrams, C.A. (1983) *Clinical Drug Therapy: Rationales for Nursing Practice,* pp. 211-228. J.B. Lippincott Co., Philadelphia.

Ales-Martinez, J.E., Cuende, E., Martinez-A., C., Parkhouse, R.M.E., Pezzi, L., Scott, D.W. (1991) Signalling in B cells. *Immunol. Today* 12(6), 201-205.

Alleyne, G.A.O., Young, V.H. (1967) Adrenocortical function in children with severe protein-calorie malnutrition. *Clin. Sci.* 33, 189-200.

Alnemri, E.S., Litwack, G. (1989) Glucocorticoid-induced lymphocytolysis is not mediated by an induced endonuclease. *J. Biol. Chem.* 264(7), 4104-4111.

Alt, F., Rosenberg, N., Lewis, S., Thomas, E., Baltimore, D. (1981) Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: rearrangement of heavy but not light chain genes. *Cell* 27, 381-390.

Antonacci, A.C., Reaves, L.E., Calvano, S.E., Amand, R., De Riesthal, H.F., Shires, G.T. (1984) Flow cytometric analysis of lymphocyte subpopulations after thermal injury in human beings. *Surg. Gynecol. Obstet.* 159(1), 1-8.

Archer, T.K., Hager, G.L., Omichinski, J.G. (1990) Sequence-specific DNA binding by glucocorticoid receptor "zinc finger peptides". *Proc. Natl. Acad. Sci. USA* 87, 7560-7564.

Armanini, D., Endres, S., Kuhnle, U., Weber, P.C. (1988) Parallel determination of mineralocorticoid and glucocorticoid receptors in T- and B-lymphocytes of human spleen. *Acta Endocrinol. (Copenh)* 118, 479-482.

Aspinall, R., Owen, J.J.T. (1983) Kinetics of sequential appearance of IgM and IgD on B lymphocytes in the bone marrow of the adult mouse. *Immunology* 48, 309-313.

Axelrod, L. (1989) Side effects of glucocorticoid therapy. *Anti-inflammatory Steroid Action. Basic and Clinical Aspects.,* pp. 377-408. Academic Press, San Diego.

Bach, J.F., Strom, R.B. (1985) Corticosteroids The Mode of Action of Immunosuppressive Agents. *Research Monographs in Immunology* 9, 21-104. Elsevier, Amsterdam.

Ballard, P.L., Carter, Janet P., Graham, Beatrice S., Baxter, John D. (1975) A radioreceptor assay for evaluation of the plasma glucocorticoid activity of natural and synthetic steroids in man. *J. Clin. Endocrinol.* 41, 290-304.

Balow, J.E., Hurley, D.L., Fauci, A.S. (1975) Immunosuppressive effects of glucoocrticoids: differential effects of acute vs chronic administration on cell mediated immunity. *J. Immunol.* 114(3), 1072-1076.

Baltimore, D. (1974) Is terminal deoxynucleotidyl transferase a somatic mutagen in lymphocytes? *Nature* 248, 409-411.

Beato, M. (1989) Gene regulation by steroid hormones. *Cell* 56, 335-344.

Beato, M., Chalepakis, G., Schauer, M., Slater, E.P. (1989) DNA regulatory elements for steroid hormones. *J. steroid Biochem.* 32(5), 737-748.

Bellingham, D.L., Cidlowski, J.A. (1988) The glucocorticoid receptor: Functional implications of protein and gene structure Steroid Receptors and Disease. *Cancer, Autoimmune, Bone, and Circulatory Disorders,* pp. 97-119. Marcel Dekker, Inc. New York

Benhhamou, L.E., Cazenave, P-A., Sarthou, P. (1990) Anti-immunoglobulins induce death by apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol.* 20, 1405-1407.

Benner, R., Van Dongen, J.J.M., Van Oudenaren, A. (1978) Corticosteroids and the humoral immune response of mice I. Differential effect of corticosteroids upon antibody formation to sheep red blood cells in spleen and bone marrow. *Cell. Immunol.* 41, 52-61.

Benner, R., Van Oudenaren, A. (1979) Corticosteroids and the humoral immune response of mice II. Enhancement of bone marrow antibody formation to lipopolysaccharide by high doses of corticosteroids. *Cell. Immunol.* 48, 267-275.

Berne, R.M., Levy, M.N. (1983) *Physiology,* p. 1043. C.V. Mosby Co. St. Louis

Besedovsky, H., Sorkin, E., Keller, M., Muller, J. (1975) Changes in blood hormone levels during the immune response. *Proc. Soc. Exp. Biol. Med.* 150, 466-470.

Besedovsky, H.O., Sorkin, E., Keller, M. (1978) Changes in the concentration of corticosterone in the blood during skin-graft rejection in the rat *J. Endocrinology* 76, 175-176.

Besedovsky, H., del Rey, A., Sorkin, E., Dinarello, C.A. (1986) Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* 233, 652-654.

Bomsztyk, K., Toivola, B., Emery, D.W., Rooney, J.W., Dower, S.K., Rachie, N.A., Sibley, C.H. (1990) Role of cAMP in interleukin-1-induced λ light chain gene expression in murine B cell line. *J. Biol. Chem.* 265(16), 9413-9417.

Bondy, P.K., Rosenberg, L.E. (1980) *Metabolic Control And Disease,* 8th edn, p. 1432. W.B. Saunders Co., Philadelphia.

Bourgeois, S., Newby, R.F. (1977) Diploid and haploid states of the glucocorticoid receptor gene of mouse lymphoid cell lines. *Cell* 11, 423-430.

Bourgeois, S., Newby, R.F. (1979) Correlation between glucocorticoid receptor and cytolytic response of murine lymphoid cell lines. *Cancer Res.* 39, 4749-4751.

Bowen, D.L., Fauci, A.S. (1984) Selective suppressive effects of glucocorticoids on the early events in the human B cell activation process. *J. Immunology* 133(4), 1885-189.

Brien, T.G. (1981) Human corticosteroid binding globulin. *Clin. Endocrinol.* 14, 193-212.

Burnstein, K.L., Cidlowski, J.A. (1989) Regulation of gene expression by glucocorticoids. *Ann. Rev. Physiol.* 51, 683-699.

Cambier, J.C. Ransom, J.T. (1987) Molecular mechanisms of transmembrane signaling in B lymphocytes. *Ann. Rev. Immunol.* 5, 175-199.

Chobert, M-N., Barouki, R., Finidori, J., Aggerbeck, M., Hanoune, J., Philibert, D., Deraedt, R. (1983) Antiglucocorticoid properties of RU 38486 in a differentiated hepatoma cell line. *Biochem. Pharmacol.* 32(22), 3481-3483.

Cidlowski, J.A., Cidlowski, N.B. (1982) Glucocorticoid receptors and the cell cycle: Evidence that the accumulation of glucocorticoid receptors during the S phase of the cell cycle is dependent on ribonucleic acid and protein synthesis. *Endocrinology* 110(5), 1653-1662.

Claman, H.N., Moorhead, H.W., Benner, W.H. (1971) Corticosteroids and lymphoid cells in vitro. I. Hydrocortison lysis of human, guinea pig, and mouse thymus cells. *J. Lab. Clin. Med.* 78(4), 499-507.

Claman, H.N. (1972) Corticosteroids and lymphoid cells. *New England J. Med.* 287(8), 388-397.

Coffman, R.L., Weissman, I.L. (1981) B220: a B cell-specific member of the T200 glycoprotein family. *Nature* 289, 681-683.

Coffman, R.L., Weissman, I.L. (1981) A monoclonal antibody that recognizes B cells and B cell precursors in mice. *J. Exp. Med.* 153, 269-279.

Coffman, R.L. (1982) Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol. Rev.* 69, 5-23.

Cohen, J.J. (1972) Thymus-derived lymphocytes sequestered in the bone marrow of hydrocortisone-treated mice. *J. Immunol.* 108(3), 841-844.

Cohen, J.J., Duke, R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunology* 132(1), 38-42.

Colbert, R.A., Young, D.A. (1986) Glucocorticoid-induced messenger ribonucleic acids in rat thymic lymphocytes: rapid primary effects specific for glucocorticoids. *Endocrinology* 119(6), 2598-2605.

Compton, M.M., Cidlowski, J.A. (1986) Rapid in vivo effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. *Endocrinology* 118(1), 38-45.

Compton, M.M., Cidlowski, J.A. (1987) Identification of a glucocorticoid-induced nuclease in thymocytes. A potential "lysis gene" product. *J. Biol. Chem.* 262(17), 8288-8292.

Compton, M.M., Haskill, S.J., Cidlowski, J.A. (1988) Analysis of glucocorticoid actions on rat thymocyte deoxyribonucleic acid by fluorescence-activated flow cytometry. *Endocrinology* 122(5), 2158-2164.

Cox, J.H., Ford, W.L. (1982) The migration of lymphocytes across specialized vascular endothelium. *Cell. Immunol.* 66, 407-422.

Crabtree, G.R., Munck, A., Smith, K.A. (1980) Glucocorticoids and Lymphocytes II. Cell cycle-dependent changes in glucocorticoid receptor content. *J. Immunol.* 125(1), 13-17.

Cupps, T.R., Edgar, L.C., Thomas, C. A., Fauci, A.S. (1984) Multiple mechanisms of B cell immunoregulation in man after administration of in vivo corticosteroids. *J. Immunol.* 132(1), 170-175.

Cupps, T.R., Gerrard, T.L., Falkoff, R.J.M., Whalen, G., Fauci, A.S. (1985) Effects of in vitro corticosteroids on B cell activation, proliferation, and differentiation. *J. Clin. Invest.* 75, 754-761.

Cupps, T.R. (1989) Effects of glucocorticoids on lymphocyte function. *Anti-Inflammatory Steroid Action. Basic and Clinical Aspects,* pp. 132-150. Academic Press, Inc. San Diego.

Dalman, F.C., Bresnick, E.H., Patel, P.D., Perdew, G.H., Watson Jr., S.J., Pratt, W.B. (1989) Direct evidence that the glucocorticoid receptor binds to hsp90 at or near the termination of receptor translation in vitro. *J. Biol. Chem.* 264(33), 19815-19821.

Danielsen, M., Hinck, L., Ringold, G.M. (1989) Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* 57, 1131-1138.

Dausse, J.P., Duval, D., Meyer, P., Gaignault, J.C., Marchandeau, C., Raynaud, J.P. (1977) The relationship between glucocorticoid structure and effects upon thymocytes. *Molec. Pharmacol.* 13, 948-955.

Deenen, G.J., van Balen, I., Opstelten, D. (1990) In rat B lymphocyte genesis sixty percent is lost from the bone marrow at the transition of nondividing pre-B cell to $sIgM^+$ B lymphocyte, the stage of Ig light chain gene expression. *Eur. J. Immunol.* 20, 557-564.

DeFranco, A.L., Gold, M.R., Jakway, J.P. (1987) B-Lymphocyte signal transduction in response to anti-immunoglobulin and bacterial lipopolysaccharide. *Immunol. Rev.* 95, 161-176.

Dennis, G.J., Mond, J.J. (1986) Corticosteroid-induced suppression of murine B cell immune response antigens. *J. Immunol*. 136(5), 1600-1604.

Dennis, G., June, C.H., Mizuguchi, J., Ohara, J., Witherspoon, K., Finkelman, F.D., McMillan, V., Mond, J.J. (1987) Glucocorticoids suppress calcium mobilization and phospholipid hydrolysis in anti-Ig antibody-stimulated B cells. *J. Immunol*. 139(8), 2516-2523.

DePasquale-Jardieu, P., Fraker, P.J. (1979) The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. *J. Nutr*. 109(11), 1847-1855.

DePasquale-Jardieu, Paula, Fraker, Pamela J. (1980) Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. *J. Immunol*. 124(6), 2650-2655.

Desiderio, S.V., Yancopoulos, G.D., Paskind, M., Thomas, E., Boss, M.A., Landau, N., Alt, F.W., Baltimore, D. (1984) Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* 311, 752-755.

Dieken, E.S., Meese, E.U., Miesfeld, R.L. (1990) nt^i Glucocorticoid receptor transcripts lack sequences encoding the amino-terminal transcriptional modulatory domain. *Molec. Cell. Biol*. 10(9), 4574-4581.

Dinarello, C.A. (1988) Biology of interleukin 1. *FASEB J.* 2, 109-115.

Di Padova, F., Pozzi, C., Tondre, M.J., Tritapepe, R. (1991) Selective and early increase of IL-1 inhibitors, IL-6 and cortisol after elective surgery. *Clin. Exp. Immunol.* 85, 137-142.

Dorshkind, K. (1986) In vitro differentiation of B lymphocytes from primitive hemopoietic precursors present in long-term bone marrow cultures. *J. Immunol.* 136(2), 422-429.

Dorshkind, Kenneth (1988) IL-1 inhibits B cell differentiation in long term bone marrow cultures. *J. Immunol.* 141(2), 531-538.

Dorshkind,Kenneth (1990) Regulation of hemopoiesis by bone marrow stromal cells and their products. *Ann. Rev. Immunol.* 8, 111-137.

Dracott, B.N., Smith, C.E.T. (1979) Hydrocortisone and the antibody response in mice I. Correlations between serum cortisol levels and cell numbers in thymus, spleen, marrow and lymph nodes. *Immunology* 38, 429-435.

Dracott, B.N., Smith, C.E.T. (1979) Hydrocortisone and the antibody response in mice II. Correlations between serum antibody and PFC in thymus, spleen, marrow and lymph nodes. *Immunology* 38, 437-443.

Duval, D., Dausse, J.P., Dardenne, M. (1976) Glucocorticoid receptors in corticosensitive and corticoresistant thymocyt subpopulations I. Characterization of glucocorticoid receptors and isolation of a corticoresistant subpopulation. *Biochim. Biophys. Acta* 151, 82-91.

Duval, D., Homo, F., Fournier, C., Dausse, J.P. (1979) Glucocorticoid receptors in mouse spleen cell subpopulations. *Cell. Immunol.* 46, 1-11.

Duvall, E., Wyllie, A.H., Morris, R.G. (1985) Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology* 56, 351-358.

Eastman-Recks, S.B., Vedeckis, W.V. (1986) Glucocorticoid inhibition of c-myc, c-myb, and c-Ki-ras expression in a mouse lymphoma cell line. *Cancer Res.* 46, 2457-2462.

Era, T., Ogawa, M., Nishikawa, S-I., Okamoto, M., Honjo, T., Akagi, K., Miyazaki, J-I., Yamamura, K-I. (1991) Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. *EMBO J.* 10(2), 337-342.

Eriksson, P., Wrange, O. (1990) Protein-protein contacts in the glucocorticoid receptor homodimer influence its DNA binding properties. *J. Biol. Chem.* 265(6), 3535-3542.

Faict, D., De Moor, P., Van Baelen, H., Heyns, W., Stevens, E., Ceuppens J. (1983) Cortisol-free transcortin: Preparation and effect on mitogen-stimulated lymphocytes. *J. Steroid Biochem.* 19(4), 1397-1401.

Faict, D., Ceuppens, J.L., De Moor, P. (1985) Transcortin modulates the effect of cortisol on mitogen-induced lymphocyte proliferation and immunoglobulin production. *J. Steroid Biochem.* 23(5A), 553-555.

Fauci, A.S., Dale, D.C. (1975) Alternate-day prednisone therapy and human lymphocyte subpopulations. *J. Clin. Invest.* 55, 22-32.

Fauci, A.S. (1975) Mechanisms of corticosteroid action on lymphocyte subpopulations I. Redistribution of circulating T and B lymphocytes to the bone marrow. *Immunology* 28, 669-680.

Fauci, A.S. (1976) Mechanisms of corticosteroid action on lymphocyte subpopulations II. Differential effects of in vivo hydrocortisone, prednisone and dexamethasone on in vitro expression of lymphocyte function. *Clin. Exp. Immunol.* 24, 54-62.

Fernandez-Ruiz, E., Rebollo, A., Nieto, M. A., Sanz, E., Somoza, C., Ramirez, F., Lopez-Rivas, A., Silva, A. (1989) IL-2 protects T cell hybrids from the cytolytic effect of glucocorticoids. Synergistic effect of IL-2 and dexamethasone in the induction of high-affinity IL-2 receptors. *J. Immunol.* 143(12), 4146-4151.

Forsthoefel, A.M., Thompson, E.A. (1987) Glucocorticoid regulation of transcription of the c-myc cellular protooncogene in P1798 cells. *Molec. Endocrinol.* 1, 899-907.

Forster, I., Rajewsky, K. (1990) The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. USA* 87, 4781-4784.

Fulop, G., Gordon, J., Osmond, D.G. (1983) Regulation of lymphocyte production in the bone marrow I. Turnover of small lymphocytes in mice depleted of B lymphocytes by treatment with anti-IgM antibodies. *J. Immunol.* 130(2), 644-648.

Gallili, U. (1983) Glucocorticoid induced cytolysis of human normal and malignant lymphocytes. *J. Steroid Biochem.* 19(1), 483-490.

Gimble, J.M., Dorheim, M-A., Cheng, Q., Medina, K., Wang, C-S., Jones, R., Koren, E., Pietrangeli, C., Kincade, P.W. (1990) Adipogenesis in a murine bone marrow stromal cell line capable of supporting B lineage lymphocyte growth and proliferation: biochemical and molecular characterization. *Eur. J. Immunol.* 20, 379-387.

Giri, J.G., Kincade, P.W., Mizel, S.B. (1984) Interleukin 1-mediated induction of κ-light chain synthesis surface immunoglobulin expression on pre-B cells. *J. Immunol.* 132(1), 223-228.

Gisler, R.H., Soderberg, A., Kamber, M. (1987) Functional maturation of murine B lymphocyte precursors II. Analysis of cells required from the bone marrow microenvironment. *J. Immunol.* 138(8), 2433-2438.

Goodwin, R.G., Lupton, S., Schmierer, A., Hjerrild, K.J., Jerzy, R., Clevenger, W., Gillis, S., Cosman, D., Namen, A.E. (1989) Human interleukin 7: Molecular cloning and growth factor activity on human and murine B-lineage cells. *Proc. Natl. Acad. Sci. USA* 86, 302-306.

Grayson, J., Dooley, N.J., Koski, I.R., Blaese, R.M. (1981) Immunoglobulin production induced in vitro by glucocorticoid hormones: T cell-dependent stimulation of immunoglobulin production without B cell proliferation in cultures of human peripheral blood lymphocytes. *J. Clin. Invest.* 68, 1539-1547.

Green, O.C., winter, R.J., Kawahara, F.S., Phillips, L.S., Lewy, P.R., Hart, R.L., Pachman, L.M. (1978) Pharmacokinetic studies of prednisolone in children. *J. Pediat.* 93(2), 299-303.

Gregoire, K.E., Goldschneider, I., Barton, R.W., Bollum F.J. (1977) Intracellular distribution of terminal deoxynucleotidyl transferase in rat bone marrow and thymus. *Proc. Natl. Acad. Sci. USA* 74(9), 3993-3996.

Gregory, C.D., Dive, C., Henderson, S., Smith, C.A., Williams, G.T., Gordon, J., Rickinson, A.B. (1991) Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* 349, 612-614.

Gu, H., Tarlinton, D., Muller, W., Rajewsky, K., Forster, I. (1991) Most peripheral B cells in mice are ligand selected. *J. Exp. Med.* 173, 1357-1371.

Hamanaka, Y., Manabe, H., Tanaka, H., Monden, Y., Uozumi, T., Matsumoto, K. (1970) Effects of surgery on plasma levels of cortisol, corticosterone and non-protein-bound-cortisol. *Acta Endrocrin.* 64, 439-451.

Hammond, G.L. (1990) Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Rev.* 11(1), 65-79.

Harbour, D.V., Chambon, P., Thompson, E. Brad (1990) Steroid mediated lysis of lymphoblasts requires the DNA binding region of the steroid hormone receptor. *J. Steroid Biochem.* 35(1), 1-9.

Hard, T., Kellenbach, E., Boelens, R., Maler, B.A., Dahlman, K., Freedman, L.P., Carlstedt-Duke, J., Yamamoto, K.R., Gustafsson, J-A., Kaptein, R. (1990) Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* 249, 157-160.

Harmon, J.M., Norman, M.R., Fowlkes, B.J., Thompson, E.B. (1979) Dexamethasone induces irreversible G_1 arrest and death of a human lymphoid cell line. *J. Cell. Physiol.* 98, 267-278.

Hasegawa,Y., Bonavida, B. (1989) Calcium-independent pathway of tumor necrosis factor-mediated lysis of target cells. *J. Immunol.* 142(8), 2670-2676.

Hayashi, J., Medlock, E.S., Goldschneider, I. (1984) A selective culture system for generating terminal deoxynucleotidyl transferase-positive (TdT^+) lymphoid precursor cells in vitro. *J. Exp. Med.* 160, 1622-1639.

Haynes, B.F., Katz, P., Fauci, A.S. (1979) Mechanisms of corticosteroid action on lymphocyte subpopulations. *Cell. Immunol.* 44, 169-178.

Henney, C.S. (1989) Interleukin 7: effects on early events in lymphopoiesis. *Immunol. Today* 10(5), 170-173.

Hermans, M.H.A., Harsuiker, H., Opstelten, D. (1989) An in situ study of B-lymphocytopoiesis in rat bone marrow: topographical arangement of terminal deoxynucleotidyl transferase-positive cells and pre-B cells *J. Immunol.* 142(1), 67-73.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., Korsmeyer, S. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348, 334-336.

Houmard, J.A., Costill, D.L., Mitchell, J.B., Park, S.H., Fink, W.J., Burns, J.M. (1990) Testosterone, cortisol, and creatine kinase levels in male distance runners during reduced training. *Int. J. Sports Med.* 11, 41-45.

Housley, P.R., Sanchez, E.R., Danielsen, M., Ringold, G.M., Pratt, W.B. (1990) Evidence that the conserved region in the steroid binding domain of the glucocortcoid receptor is required for both optimal binding of hsp90 and protection from proteolytic cleavage. *J. Biol. Chem.* 265(22), 12778-12781.

Hsu, B.R-S., Siiteri, P.K., Kuhn, R.W. (1986) Interactions between corticosteroid-binding globulin (CBG) and target tissues *Binding Proteins of Steroid Hormones* 149, 577-591.

Hutchison, K.A., Matic, G., Meshinchi, S., Bresnick, E.H., Pratt, W.B. (1991) Redox manipulation of DNA binding activity and BuGR eptiope reactivity of the glucocorticoid receptor. *J. Biol. Chem.* 266(16), 10505-10509.

Iglesias, A., Kopf, M., Williams, G.S., Buhler, B., Kohler, G. (1991) Molecular requirements for the μ-induced light chain gene rearrangement in pre-B cells. *EMBO J.* 10(8), 2147-2156.

Jacobsen, K., Tepper, J., Osmond, D.G. (1990) Early B-lymphocyte precursor cells in mouse bone marrow: Subosteal localization of $B220^+$ cells during postirradiation regeneration. *Exp. Hematol*. 18, 304-310.

Kagan, R.J., Bratescu, A., Jonasson, O., Matsuda, T., Teodorescu, M. (1989) The relationship between the percentage of circulating B cells, corticosteroid levels, and other immunologic parameters in thermally injured patients. *J. Trauma* 29(2), 208-213.

Karasuyama, H., Kudo, A., Melchers, F. (1990) The proteins encoded by the VpreB and λ5 pre-B cell-specific gene can associate with each other and with μ heavy chain. *J. Exp. Med*. 172, 969-972.

Kayganich, K., Watson, J.T., Kilts, C., Ritchie, J. (1990) Determination of plasma dexamethasone by chemical oxidation and electron capture negative ionization mass spectrometry. *Biomed. Environ. Mass Spec*. 19, 341-347.

Kearney, J.F., Cooper, M.D. Klein, J., Abney, E.R., Parkhouse, R.M.E., Lawton, A.R. (1977) Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. *J. Exp. Med*. 146, 297-301.

Kerr, J.F.R., Searle, J., Harmon, B.V., Bishop, C.J. (1987) Apoptosis. *Perspectives on Cell Death,* pp. 93-128. Oxford University Press, Oxford.

Kierney, P.C., Dorshkind, K. (1987) B lymphocyte precursors and myeloid progenitors survive in diffusion chamber cultures but B cell differentiation requires close association with stromal cells. *Blood* 70(5), 1418-1424.

Kincade, P.W., Lee, G., Watanabe, T., Sun, L., Scheid, M.P. (1981) Antigens displayed on murine B lymphocyte precursors. *J. Immunol.* 127(6), 2262-2268.

Kincade, P.W., Lee, G., Pietrangeli, C.E., Hayashi, S-I., Gimble, J.M. (1989) Cells and molecules that regulate B lymphopoiesis in bone marrow. *Ann. Rev. Immunol.* 7, 111-143.

King, A.G., Wierda, D., Landreth, K.S. (1988) Bone marrow stromal cell regulation of B-lymphoiesis I. The role of macrophages, IL-1, and IL-4 in pre-B cell maturation. *J. Immunol.* 141(6), 2016-2026.

Kizaki, H., Tadakuma, T., Odaka, C., Muramatsu, J., Ishimura, Y. (1989) Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. *J. Immunol.* 143(6), 1790-1794.

Ku, G. and Owen N.W. (1986) Corticosteroid-resistant bone marrow-derived B lymphocyte progenitor for long term in vitro cultures. *J. Immunol.* 137(9), 2802-2807.

Labarca, C., Paigen, K. (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102, 344-352.

Lala, P.K., Layton, J.E., Nossal, G.J.V. (1979) Maturation of B lymphcytes II. Sequential appearance of increasing IgM and IgD in the adult bone marrow. *Eur. J. Immunol.* 9, 39-44.

Landreth, K.S., Rosse, C., Clagett, J. (1981) Myelogenous production and maturation of B lymphocytes in the mouse. *J. Immunol.* 127(5), 2027-2034.

Landreth, K.S., Kincade, P.W., Lee, G., Medlock, E.S. (1983) Phenotypic and functional characterization of murine B lymphocyte precursors isolated from fetal and adult tissues. *J. Immunol.* 131(2), 572-580.

Lapointe, M.C., Baxter, J.D. (1989) Molecular biology of glucocorticoid hormone action. *Anti-inflammatory Steroid Action. Basic and Clinical Aspects,* pp. 3-29. Academic Press, San Diego.

Lee, G., Namen, A.E., Gillis, S., Ellingsworth, L.R., Kincade, P.W. (1989) Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β. *J. Immunol.* 142(11), 3875-3883.

Levine, M.A., Claman, H.N. (1970) Bone marrow and spleen: Dissociation of immunologic properties by cortisone. *Science* 167, 1515-1517.

Levitt, D., Cooper, M.D. (1980) Mouse pre-B cells synthesize and secrete μ heavy chains but not light chains. *Cell* 19, 617-625.

Lippman, M.E., Perry, S., Thompson, E.B. (1974) Cytoplasmic glucocorticoid-binding proteins in glucocorticoid-unresponsive human and mouse leukemic cell lines. *Cancer Res*. 34, 1572-1576.

Liu, Y-J., Joshua, D.E., Williams, G.T., Smith, C.A., Gordon, J., MacLennan, I.C.M. (1989) Mechanism of antigen-driven selection in germinal centres. *Nature* 342, 929-931.

Liu, Y-J., Cairns, J.A., Holder, M.J., Abbot, S.D., Jansen, K.U., Bonnefoy, J-Y., Gordon, J., MacLennen, I.C.M. (1991) Recombinant 25-kDa CD23 and interleukin 1α promote the survival of germinal centre B cells: evidence for bifurcation in the development of centrocytes rescued from apoptosis. *Eur. J. Immunol*. 21, 1107-1114

Luster, M.I., Germolec, D.R., Clark, G., Wiegand, G., Rosenthal, G.J. (1988) Selective effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and corticosteroid on in vitro lymphocyte maturation. *J. Immunol*. 140(3), 928-935.

MacDonald, H.R., Lees, R.K. (1990) Programmed death of autoreactive thymocytes. *Nature* 343, 642-644.

Martins, V.R., Birolli, M.I.A., Duarte, A.J.S., Brentani, M.M. (1987) Glucocorticoid receptors in subpopulations of human lymphocytes defined by monoclonal antibodies. *Cell. Immunol*. 105, 443-446.

Mattingly, D. (1962) A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. *J. Clin. Path.* 15, 374-379.

McConkey, D.J., Hartzell, P., Nicotera, P., Orrenius, S. (1989) Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB J.* 3, 1843-1849.

McConkey, D.J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A.H., Orrenius, S. (1989) Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. *Arch. Biochem. Biophys.* 269(1), 365-370.

McConkey, D.J., Hartzell, P., Jondal, M., Orrenius, S. (1989) Inhibition of DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. *J. Biol. Chem.* 264(23), 13399-13402.

McConkey, D.J., Hartzell, P., Chow, S.C., Orrenius, S., Jondal, M. (1989) Interleukin 1 inhibits T cell receptor-mediated apoptosis in immature thymocytes. *J. Biol. Chem.* 265(6), 3009-3011.

McConkey, D.J., Orrenius, S., Jondal, M. (1990) Cellular signalling in programmed cell death (apoptosis). *Immunol. Today* 11(4), 120-121.

McConkey, D.J., Hartzell, P., Orrenius, S. (1990) Rapid turnover of endogenous endonuclease activity in thymocytes: effects of inhibitors of macromolecular synthesis. *Arch. Biochem. Biophys.* 278(1), 284-287.

McConkey, D.J., Aguilar-Santelises, M., Hartzell, P., Eriksson, I., Mellstedt, H., Orrenius, S., Jondal, M. (1991) Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. *J. Immunol.* 146(3), 1072-1076.

McMillan, V.M., Dennis, G.J., Glimcher, L.H., Finkelman, F.D., Mond, J.J. (1988) Corticosteroid induction of $Ig^{+}Ia^{-}$ B cells in vitro is mediated via interaction with the glucocorticoid cytoplasmic receptor. *J. Immunol.* 140(8), 2549-2555.

Medina, C.A., Li, G., Fraker, P.J. (1988) Improved plaque production for short-term bone marrow and fetal liver cultures. *J. Immunol. Methods* 111, 233-240.

Mendel, D.B., Bodwell, J.E., Gametchu, B., Harrison, R.W., Munck, A. (1986) Molybdate-stabilized nonactivated glucocorticoid-receptor complexes contain a 90-kDa non-steroid-binding phosphoprotein that is lost on activation. *J. Biol. Chem.* 261(8), 3758-3763.

Miesfeld, R., Rusconi, S., Godowski, P.J., Maler, B.A., Okret, S., Wikstom, A-C., Guctafsson, J-A., Yamamoto, K.R. (1986) Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. *Cell* 46, 389-399.

Miller, S.C., Osmond, D.G. (1975) Lymphocyte populations in mouse bone marrow: Quantitative kinetic studies in young, pubertal and adult C3H mice. *Cell Tissue Kinet.* 8, 97-110.

Miyake, K, Medina, K.L., Hayashi, S-I., Ono, S., Hamaoka, T., Kincade, P.W. (1990) Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J. Exp. Med.* 171(2), 477-488.

Miyake, K., Medina, K., Ishihara, K., Kimoto, M., Auerbach, R., Kincade, P.W. (1991) A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *J. Cell Biol.* 114(3), 557-565.

Miyake, K., Weissman, I.L., Greenberger, J.S., Kincade, P.W. (1991) Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* 173, 599.

Moguilewsky, M. Philibert, D. (1984) RU 38486: Potent anitglucocrticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation. *J. Steroid Biochem.* 20(1), 271-276.

Morrissey, P.J., Conlon, P., Braddy, S., Williams, D.E., Namen, A.E., Mochizuki, D.Y. (1991) Administration of IL-7 to mice with cyclophosphamide-induced lymphopenia accelerates lymphocyte repopulation. *J. Immunol.* 146(5), 1547-1552.

Morrissey, P.J., Conlon, P., Charrier, K., Braddy, S., Alpert, A., Williams, D., Namen, A.E., Mochizuki, D. (1991) Administration of IL-7 to normal mice stimulates B-lymphopoiesis and peripheral lymphadenopathy. *J. Immunol*. 147(2), 561-568.

Munck, A., Brinck-Johnsen, T. (1968) Specific and nonspecific physicochemical interactions of glucocorticoids and related steroids with rat thymus cells in vitro. *J. Biol. Chem*. 243(21), 5556-5565.

Namen, A.E., Schmierer, A.E., March, C.J., Overell, R.W., Park, L.S., Urdal, D.L., Mochizuki, D.Y. (1988) B cell precursor growth-promoting activity: Purification and characterization of a growth factor active on lymphocyte pecursors. *J. Exp. Med*. 167, 988-1002.

Namen, A.E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D.Y., Schmierer, A., Mosley, B., March, C.J., Urdal, D., Gillis, S., Cosman, D., Goodwin, R.G. (1988) Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature (London)* 333, 571-573.

Neiman, P.E., Thomas, S.J., Loring, G. (1991) Induction of apoptosis during normal and neoplastic B-cell development in the bursa of Fabricius. *Proc. Natl. Acad. Sci. USA* 88, 5857-5861.

Nieto, M.A., Lopez-Rivas, A. (1989) IL-2 protects T lymphocytes from glucocorticoid-induced DNA fragmentation and cell death. *J. Immunol*. 143(12), 4166-4170.

Ojeda, F., Guarda, M.I., Maldonado, C., Folch, H. (1990) Protein kinase-C involvement in thymocyte apoptosis induced by hydrocortisone. *Cell. Immunol.* 125, 535-539.

O'Mahony, J.B., Wood, J.J., Rodrick, M.L., Mannick, J.A. (1985) Changes in T lymphocyte subsets following injury. Assessment by flow cytometry and relationship to sepsis. *Ann. Surg.* 202, 580-586.

Opstelten, D., Osmond, D.G. (1983) Pre-B cells in mouse bone marrow: Immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ-chain-bearing cells in normal mice. *J. Immunol.* 131(6), 2365-2640.

Opstelten, D., Osmond, D.G. (1985) Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. *Eur. J. Immunol.* 15, 599-605.

Opstelten, D., Keenen, G.J., Rozing, J., Hunt, S.V. (1986) B lymphocyte-associated antigens on terminal deoxynucleotidyl transferase-positive cells and pre-B cells in bone marrow of the rat. *J. Immunol.* 137(1), 76-84.

Orson, F.M., Auzenne, C.A. (1988) Glucocorticosteroid-induced immunoglobulin production requires intimate contact between B cell and monocytes. *Cell. Immunol.* 112, 147-155.

Osmond, D.G., Nossal, G.J.V. (1974) Differentiation of lymphocytes in mouse bone marrow II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. *Cell. Immunol.* 13, 132-145.

Osmond, D.G., Owen, J.J.T. (1984) Pre-B cells in bone marrow: size distribution profile, proliferative capacity and peanut agglutinin binding of cytoplasmic μ chain-bearing cell populations in normal and regenerating bone marrow. *Immunology* 51, 333-342.

Ostergaard, H.L., Trowbridge, I.S. (1991) Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells. *Science* 253, 1423-1425.

Owens, G.P., Hahn, W.E., Cohen, J.J. (1991) Identification of mRNAs associated with programmed cell death in immature thymocytes. *Molec. Cell. Biol.* 11(8), 4177-4188.

Paige, C.J., Kincade, P.W., Ralph, P. (1978) Murine B cell leukemia line with inducible surface immunoglobulin expression. *J. Immunol.* 121(2), 641-647.

Page, D.M., DeFranco, A.L. (1990) Antigen receptor-induced cell cycle arrest in WEHI-231 B lymphoma cells depends on the duration of signalling before the G_1 restriction point. *Molec. Cell. Biol.* 10(6), 3003-3012.

Park, Y-H., Osmond, D.G. (1987) Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. *J. Exp. Med.* 165, 444-458.

Park, Y-H., Osmond, D.G. (1989) Post-irradiation regeneration of early B-lymphocyte precursor cells in mouse bone marrow. *Immunology* 66, 343-347.

Park, Y-H., Osmond, D.G. (1989) Dynamics of early B lymphocyte precursor cells in mouse bone marrow: proliferation of cells containing terminal deoxynucleotidyl transferase. *Eur. J. Immunol.* 19, 2139-2144.

Park, Y.H., Osmond, D.G. (1991) Regulation of early precursor B cell proliferation in mouse bone marrow: stimulation by exogenous agents mediated by macrophages in the spleen. *Cell. Immunol.* 135, 168-183.

Pechatnikov, V.A., Afanasyev, V.N., Korol, B.A., Korneev, V.N., Rochev, Yu. A., Umansky, S.R. (1986) Flow cytometry analysis of DNA degradation in thymocytes of γ-irradiated or hydrocortisone treated rats. *Gen. Physiol. Biophys.* 5, 273-284.

Peschel, C., Green, I., Paul, W.E. (1989) Preferential proliferation of immature B lineage cells in long-term stromal cell-dependent cultures with IL-4. *J. Immunol.* 142(5), 1558-1568.

Picard, D., Yamamoto, K.R. (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.* 6(11), 3333-3340.

Picard, D., Khursheed, B., Garabedian, M.J., Gortin, M.G., Lindquist, S., Yamamoto, K.R. (1990) Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* 348, 166-168.

Pietrangeli, C.E., Osmond, D.G. (1987) Regulation of B-lymphocyte production in the bone marrow: mediation of the effects of exogenous stimulants by adoptively transferred spleen cells. *Cell. Immunol.* 107, 348-357.

Pleiman, C.M., Gimpel, S.D., Park, L.S., Harada, H., Taniguchi, T., Ziegler, S.F. (1991) Organization of the murine and human interleukin-7 receptor genes: Two mRNAs generated by differential splicing and presence of a type I-interferon-inducible promoter. *Molec. Cell. Biol.* 11(6), 3052-3059.

Press, O.W., Rosse, C., Clagett, J. (1977) The distribution of rapidly and slowly renewed T, B, and "null" lymphocytes in mouse bone marrow, thymus, lymph nodes, and spleen. *Cell. Immunol.* 33, 114-124.

Raff, M.C., Megson, M., Owen, J.J.T., Cooper, M.D. (1976) Early production of intracellular IgM by B-lymphocyte precursors in mouse. *Nature* 259, 224-226.

Ranelletti, F.O., Piantelli, M., Iacobelli, S., Musiani, P., Longo, P., Lauriola, L., Marchetti, P. (1981) Glucocorticoid receptors and in vitro corticosensitivity of peanut-positive and peanut-negative human thymocyte subpopulations. *J. Immunol.* 127(3), 849-855.

Rees, A.J., Lockwood, C.M. (1982) Immunosuppressive drugs in clinical practice. *Clinical Aspects of Immunology,* pp. 507-564. Blackwell Scientific Publications, Oxford.

Rennick, D., Yang, G., Muller-Sieburg, C., Smith, C., Arai, N., Takabe, Y., Gemmell, L. (1987) Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. *Proc. Natl. Acad. Sci. USA* 84, 6889-6893.

Reynolds, J.E.F. (1989) *Martindale: The Extra Pharmacopoeia,* 29th edn, pp. 872-901. The Pharmaceutical Press, London.

Rodriguez-Tarduchy, G., Collins, M., Lopez-Rivas, A. (1990) Regulation of apoptosis in interleukin-3-dependent hemopoietic cells by interleukin-3 and calcium ionophores. *EMBO J.* 9(9), 2997-3002.

Roess, D.A., Bellone, C.J., Ruh, M.F., Nadel, E.M., Ruh, T.S. (1982) The effects of glucocorticoids on mitogen-stimulated B-lymphocytes: Thymidine incorporation and antibody secretion. *Endocrinology* 110(1), 169-175.

Roess, D.A., Ruh, M.F., Bellone, C.J., Ruh, T.S. (1983) Glucocorticoid effects on mitogen-stimulated isolated murine B-lymphocytes. *Endorcrinology* 112(4), 1530-1537.

Rolink, A., Kudo, A., Karasuyama, H., Kikuchi, Y., Melchers, F. (1991) Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cell in vitro and in vivo. *EMBO J.* 10(2), 327-336.

Rolink, A., Melchers, F. (1991) Molecular and celular origins of B lymphocyte diversity. *Cell* 66, 1081-1094.

Rosner, W., Hochberg, R. (1972) Corticosteroid-binding globulin in the rat: Isolation and studies of its influence on cortisol action in vivo. *Endocrinology* 91, 626-632.

Rosner, W., Kahn, M.S., Romas, N.A., Hyrb, D.J. (1986) Interactions of plasma steroid-binding proteins with cell membranes. *Binding Proteins of Steroid Hormones* 149, 567-575.

Rosner, W. (1990) The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocrine Rev.* 11(1), 80-91.

Sabbele, N.R., Van Oudenaren, A., Hooijkaas, H., Benner, R. (1987) The effect of corticosteroids upon murine B cells in vivo and in vitro as determined in the LPS-culture system. *Immunology* 62, 285-290.

Sabourin, L.A., Hawley, R.G. (1990) Suppression of programmed death and G_1 arrest in B-cell hybridomas by interleukin-6 is not accompanied by altered expression of immediate early respons genes. *J. Cell Physiol.* 145, 564-574.

Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* 83, 865-875.

Savill, J., Dransfield, I., Hogg, N., Haslett, C. (1990) Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature* 343, 170-173.

Saxon, A., Stevens, R.H., Ramer, S.J., Clements, P.J., Yu, D.T.Y. (1978) Glucocorticoids administered in vivo inhibit human suppressor T lymphocyte function and diminish B lymphocyte responsiveness in in vitro immunoglobulin synthesis. *J. Clin. Invest.* 61, 922-930.

Scher, I., Berning A.K., Kessler, S., Finkelman, F.D. (1980) Development of B lymphocytes in the mouse; studies of the frequency and distribution of surface IgM and IgD in normal and immune-defective CBA/N F_1 mice. *J. Immunol.* 125(4), 1686-1693.

Schlechte, J.A., Ginsberg, B.H., Sherman, B.M. (1982) Regulation of the glucocorticoid receptor in human lymphocytes. *J. Steroid Biochem.* 16, 69-74.

Schrader, J.W., Goldschneider, I., Bollum, F.J., Schrader, S. (1979) In vitro studies on lymphocyte differentiation II. Generation of terminal deoxynucleotidyl transferase-positive cells in long term cultures of mouse bone marrow. *J. Immunol.* 122(6), 2337-2339.

Schuurs, A.H.W.M., Verheul, H.A.M. (1990) Effects of gender and sex steroids on the immune response. *J. Steroid Biochem.* 35(2), 157-172.

Schwartzman, R.A., Cidlowski, J.A. (1991) Internucleosomal deoxyribonucleic acid cleavage activity in apoptotic thymocytes: Detection and endocrine regulation. *Endocrinology* 128(2), 1190-1197.

Screpanti, I., Morrone, S., Meco, D., Santoni, A., Gulino, A., Paolini, R., Crisanti, A., Mathieson, B.J., Frati, L. (1989) Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation: Effects of 17 β-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2 receptor. *J. Immunol.* 142(10), 3378-3383.

Sellins, K.S., Cohen, J.J. (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* 139(10), 3199-3206.

Shek, P.N., Sabiston, B.H. (1983) Neuroendocrine regulation of immune processes: Change in circulating corticosterone levels induced by the primary antibody response in mice. *Int. J. Immunopharmac.* 5(1), 23-33.

Shieh, J-H., Peterson, R.H.F., Moore, M.A.S. (1991) Granulocyte colony-stimulationg factor modulation of cytokine receptors on murine bone marrow cells. In vivo and in vitro studies. *J. Immunol.* 147(9), 2984-2990.

Sierakowski, S., Goodwin, J.S. (1988) Mechanism of action of glucocorticoid-induced immunoglobulin production: II. Requirement for fetal calf serum. *Clin. Exp. Immunol.* 71, 198-201.

Simons, A., Zharhary, D. (1989) The role of IL-4 in the generation of B lymphocytes in the bone marrow. *J. Immunol*. 143(8), 2540-2545.

Simpson, E.R., Waterman, M.R. (1988) Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Ann. Rev. Physiol*. 50, 427-440.

Smith, K.A., Crabtree, G.R., Kennedy, S.J., Munck, A.U. (1977) Glucocorticoid receptors and glucocorticoid sensitivity of mitogen stimulated and unstimulated human lymphocytes. *Nature* 267, 523-526.

Smith, I.F., Latham, M.C., Azubuike, J.A., Butler, W.R., Phillips, L.S., Pond, W.G., Enwonwu, C.O. (1981) Blood plasma levels of cortisol, insulin, growth hormone and somatomedin in children with marasmus, kwasiorkor, and intermediate forms of protein-energy malnutrition. *Proc. Soc. Exp. Biol. Med*. 167, 607-611.

Smith, E.L., Hill R.L., Lehman, I.R., Lefkowitz, R.J., Handler, P., White, A. (1983) *Principles of Biochemistry: Mammalian Biochemistry,* p.543. McGraw-Hill Book Co., New York.

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., Owen, J.J.T. (1989) Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* 337, 181-184.

Spackman, D.H., Riley, V. (1978) Corticosterone concentrations in the mouse. *Science* 200, 87.

Stevenson, J.R., Taylor, R. (1988) Effects of glucocorticoid and antiglucocorticoid hormones on leukocyte numbers and function. *Int. J. Immunopharmac.* 10(1), 1-6.

Sudo, T., Ito, M., Ogawa, Y., Iizuka, M., Kodama, H., Kunisada, T., Hayashi, S-I., Ogawa, M., Sakai, K., Nishikawa, S., Nishikawa, S-I. (1989) Interleukin 7 production and function in stromal cell-dependent B cell development. *J. Exp. Med.* 170, 333-338.

Sugiyama, H., Akira, S., Yoshida, N., Kishimoto, S., Yamamura, Y., Kincade, P., Honjo, T., Kishimoto, T. (1982) Relationship between the rearrangement of immunoglobulin genes, the appearance of a B lymphocyte antigen, and immunoglobulin synthesis in murine pre-B cell lines. *J. Immunol.* 126(6), 2793-2797.

Swat, W., Ignatowicz, L., Kisielow, P. (1991) Detection of apoptosis of immature $CD4^{+}8^{+}$ thymocytes by flow cytometry. *J. Immunol. Methods* 137, 79-87.

Szefler, S.J. (1989) General pharmacology of glucocorticoids. *Anti-inflammatory Steroid Action. Basic and Clinical Aspects,* pp. 353-372. Academic Press, San Diego.

Szekeres-Bartho, J., Autran, B., Debre, P., Andreu, G., Denver, L., Chaouat, G. (1989) Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. *Cell. Immunol.* 122, 281-294.

Telford, W.G., King, L.E., Fraker, P.J. (1991) Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flow cytometry. *Cell Prolif*. 24, 447-460.

Thomas, M.L., Lefrancois, L. (1988) Differential expression of the leucocyte-common antigen family. *Immunol. Today* 9, 320-326.

Thomas, M.L. (1989) The leukocyte common antigen family. *Ann. Rev. Immunol*. 7, 339-369.

Tsubata, T., Reth, M. (1990) The products of pre-B cell-specific genes (λ5 and VpreB) and the immunoglobulin μ chain form a complex that is transported onto the cell surface. *J. Exp. Med*. 172, 973-976.

Ucker, D.S. (1987) Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide process in target cells. *Nature* 327, 62-64.

Ucker, D.S., Ashwell, J.D., Nickas, G. (1989) Activation-driven cell death I. Requirements for de novo transcription and translation and association with genome fragmentation. *J. Immunol*. 143(11), 3461-3469.

Umansky, S.R., Korol, B.A., Nelipovich, P.A. (1981) In vivo DNA degradation in thymocytes of g-irradiated or hydrocortisone-treated rats. *Biochim. Biophys. Acta* 655, 9-17.

Vaux, D.L., Cory, S., Adams, J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature (London)* 335, 440-442.

Vallee, B.L., Coleman, J.E., Auld, D.S. (1991) Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc. Natl. Acad. Sci. USA* 88, 999-1003.

Van der Meer, J.W.M., Van de Gevel, J.S., Van Furth, J. (1986) The effect of glucocorticosteroids on bone marrow mononuclear phagocytes in culture *Immunobiol*. 172, 143-150.

Vedeckis, W.V., Bradshaw Jr., H.D. (1983) DNA fragmentation in S49 lymphoma cell killed with glucocorticoids and other agents. *Molec. Cell. Endocrinol*. 30, 215-227.

Velardi, A., Cooper, M.D. (1984) An immunofluorescence analysis of the ontogeny of myeloid, T, and B lineage cells in mouse hemopoietic tissues. *J. Immunol*. 133(2), 672-677.

Vermeulen, A. (1986) TeBG and CBG as an index of endocrine function. *Binding Proteins of Steroid Hormones* 149, 383-396.

Vines, R.L., Coleman, M.S., Hutton, J.J. (1980) Reappearance of terminal deoxynucleotidyl transferase containing cells in rat bone marrow following corticosteroid administration. *Blood* 56(3), 501-509.

Volenec, F.J., Wood, G.W., Mani, M.M., Robinson, D.W., Humphrey, L.J. (1979) Mononuclear cell analysis of peripheral blood from burn patients. *J. Trauma* 19(2), 86-93.

Wallace, D.M. (1987) *Methods in Enzymology: Guide to Molecular Cloning* 152, 33-39. Academic Press, San Diego.

Watson, J.T., Kayganich, K. (1989) Novel sample preparation for analysis by electron capture negative ionization mass spectrometry. *Biochem. Soc. Trans.* 17, 254-257.

Weiss, L. (1981) Haemopoiesis in mammalian bone marrow. *Micreoenvironments in Haemopoietic and Lymphoid Differentiation. CIBA Foundation Symposium* 84, 5-21. Pitman, London.

Weissman, I.L. (1973) Thymus cell maturation: Studies on the origin of cortisone-resistant thymic lymphocytes. *J. Exp. Med.* 137, 504-510.

Whitlock, C.A., Robertson, D., Witte, O.N. (1984) Murine B cell lymphopoiesis in long term culture. *J. Immunol. Methods* 67, 353-369.

Whitlock, C.A., Tidmarsh, G.F., Muller-Sieburg, C., Weissman, I.L. (1987) Bone marrow stromal cell lines with lymphopoietic activity express high levels of a pre-B neoplasia-associated molecule. *Cell* 48, 1009-1021.

Williams, D.E., Namen, A.E., Mochizuki, D.Y., Overell, R.W. (1990) Clonal growth of murine pre-B colony-forming cells and their targeted infection by a retroviral vector: dependence on interleukin-7. *Blood* 4 75(5), 1132-1138.

Wing, E.J., Magee, D.M., Barczynski, L.K. (1988) Acute starvation in mice reduces the number of T cells and suppresses the development of T-cell-mediated immunity. *Immunology* 63, 677-682.

Wira, C.R., Sandoe, C.P., Steele, M.G. (1990) Glucocorticoid regulation of the humoral immune system I. In vivo effects of dexamethasone on IgA and IgG in serum and at mucosal surfaces. *J. Immunol.* 144(1), 142-146.

Wyllie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284, 555-556.

Wyllie, A.H., Kerr, J.F.R., Currie, A.R. (1980) Cell death: The significance of apoptosis. *Int. Rev. Cytol.* 68, 251-306.

Wyllie, A.H., Morris, R.G. (1982) Hormone-induced cell death: Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am. J. Pathol.* 109, 78-87.

Wysocki, L.J., Sato, V.L. (1978) "Panning" for lymphocytes: A method for cell selection. *Proc. Natl. Acad. Sci.* 75(6), 2844-2848.

Yancopoulos, G.D., Alt, F.W. (1986) Regulation of the assembly and expression of variable-region genes. *Ann. Rev. Immunol.* 4, 339-368.

Yang, W.C., Miller, S.C., Osmond, D.G. (1978) Maturation of bone marrow lymphocytes II. Development of Fc and complement receptors and surface immunoglobulin studied by rosetting and radioautography. *J. Exp. Med.* 148, 1251-1270.

Yu, D.T.Y., Clements, P.J., Paulus, H.E., Peter, J.B., Levy, J., Barnett, E.V. (1974) Human lymphcyte subpopulations: Effect of corticosteroids *J. Clin. Invest.* 53, 565-571.

Yuh, Y-S., Thompson, E.B. (1989) Glucocorticoid effect on oncogene/growth gene expression in human T lymphoblastic leukemic cell line CCRF-CEM. *J. Biol. Chem.* 264(18), 10904-10910.

Zarbo, R.J., Visscher, D.W., Crissman, J.D. (1989) Two-color multiparametric method for flow cytometric DNA analysis of carcinomas using staining for cytokeratin and leukocyte-common antigen. *Anal. Quant. Cytol. Histol.* 11(6), 391-402.

APPENDIX

EXERCISE AND IMMUNE FUNCTION: A REVIEW

Chronic exercise has been shown to increase the overall cardiovascular health of individuals. While it is attractive to suggest that exercise also enhances host defense, until recently there has been limited information regarding the effects of exercise on immune function. Generally it has been found that moderate exercise improves some immune functions while exhausting exercise or overtraining has a deterimental effect (Fitzgerald, 1988; Fitzgerald, 1991; Pedersen *et al.*, 1991). Although much of the literature is confounded by the many different exercise protocols and training methods employed, some general conclusions can be gleaned from the recent studies addressing this issue. There have been two basic approaches to the study of the effects of exercise on immune function. One is to look at the effects of an acute bout of exercise on various immune system parameters. Many of these are human studies which use peripheral blood leukocytes for examining immune function. The second is to look at the effects of prolonged training regimens on immune function. More animal models are used in these studies but human studies also have been performed. The obvious advantage of animal studies is that immune organs other than peripheral blood are readily available for study. However, it is not yet clear if these animal studies can be generalized to humans. This review will examine both the effects of acute and chronic exercise on immune function in humans and rodents.

Acute Exercise and Immune Function

Several investigators have reported a transient leukocytosis in human peripheral blood immediately following a single bout of moderate exercise (Edwards *et al.*, 1984; Hedfors *et al.*, 1976; Nieman *et al.*, 1991; MacNeil *et al.*, 1991). Five minutes of running up and down stairs resulted in a 2-fold increase in peripheral blood lymphocytes, specifically T suppressor/cytotoxic cells along with a 4-fold increase in natural killer (NK) cells (Edwards *et al.*, 1984) which are large granular lymphocytes whose apparent role is to kill virus-infected cells and some tumor cells. A 45-minute walk at 60% VO_{2max} also resulted in leukocytosis due primarily to a significant increase in neutrophils (Nieman *et al.*, 1991). However, as previously reported, T suppressor/cytotoxic cells and NK cells also were increased in the peripheral blood (Nieman *et al.*, 1991). For example, cycling for 60 minutes at 75% VO_{2max} resulted in increased NK cells in the human peripheral blood (Kappel *et al.*, 1991; Haahr *et al.*, 1991).

Functional studies have revealed that moderate exercise causes variable responses of peripheral blood mononuclear cells to mitogens. Five minutes of stair running or a

45-minute walk at 60% VO_{2max} had no effect on the response to concanavalin A (con A) (Edwards *et al.,* 1984; Nehlsen-Cannarella *et al.,* 1991a). However, cycling at various submaximal intensities for durations of 10 to 120 minutes resulted in depressed responses to con A (MacNeil *et al.*, 1991; Hedfors *et al.*, 1976). Cycling for 10 minutes at a heart rate of 150 beats per minute caused a small but statistically significant depression in peripheral blood mononuclear cell response to phytohemagglutinin, pokeweed mitogen, and protein purified derivative of tuberculin, while the response to lipopolysaccharide was unchanged (Hedfors *et al.*, 1976). Nehlsen-Cannarella *et al.*, (1991) also reported a trend toward depressed response to phytohemagglutinin immediately after 45 minutes of walking. These changes were all transient with the subject recovering within hours after the end of exercise. It is unclear why there was a depression in the mitogen response in spite of the fact that lymphocytes increased in number in peripheral blood. It is tempting to tie cortisol levels to the depressed immune function; however, plasma cortisol concentrations were not changed by a 45-minute walk and were increased but not significantly by cycling at submaximal intensities (MacNeil *et al.*, 1991; Nehlsen-Cannarella *et al.*, 1991).

NK cell mediated lysis of target cells was significantly increased by cycling for 60 minutes at 75% VO_{2max} or by running stairs for five minutes, but the subjects returned to baseline levels within 2 hours after exercise (Kappel *et al.*, 1991; Edwards *et al.*, 1984). Interestingly, infusion of epinephrine to cause plasma concentrations similar to those seen during exercise also caused a transient increase in the number of peripheral blood NK cells along with increased NK cell activity (Kappel *et al.*, 1991). Catecholamines may play an important role in the regulation of leukocyte function during exercise since lymphocytes have adrenergic receptors (Weicker & Werle, 1991).

Exhaustive exercise has been reported to cause leukocytosis similar to that reported for submaximal exercise; however, after long-distance runs, such as marathons, there was reduced or no lymphocytosis reported (Eskola *et al.*, 1978; Nieman *et al.*, 1989; Moorthy *et al.*, 1978). The increased number of leukocytes was caused by a 4-fold increase in the number of granulocytes (Nieman *et al.*, 1989; Eskola *et al.*, 1978; Moorthy *et al.*, 1978). Further, marathon running (less than 3 hours) caused a transient suppression in the peripheral blood lymphocyte response to phytohemagglutinin, concanavalin A, and a purified protein derivative of tuberculin (Eskola *et al.*, 1978). Unlike what is observed during submaximal forms of exercise, plasma cortisol is clearly increased by marathon running (Nieman *et al.*, 1989; Eskola *et al.*, 1978; Moorthy *et al.*, 1978) and may be responsible for the lack of lymphocytosis which is seen during bouts of more moderate exercise intensities. Exhaustive exercise of shorter duration than marathon running resulted in delayed granulocytosis, peaking around 4 hours after exercise (Hansen *et al.,* 1991; Eskola *et al.*, 1978). Hansen *et al.* (1991) reported that running at near maximal speeds for 1.7, 4.8, or 10.5 km resulted in increased plasma lymphocytes during exercise followed by a loss of lymphocytes below baseline levels 2-4 hours later. They attributed this loss to increased cortisol levels which peaked 30 minutes after exercise. However, Eskola *et al.* (1978) found that after a 7-km run at more moderate speeds there was no change in the number of peripheral blood lymphocytes and there were normal responses to mitogens. The differences between these findings could be related to the fitness levels of the subjects, the intensity of exercise, and/or the environmental conditions of the run. While Hansen *et al.* (1991) used moderately trained subjects in a laboratory setting, Eskola *et al.* (1978) used more highly trained subjects and did not indicate the environmental conditions of the exercise test.

As has been reported for acute submaximal exercise, exercise to exhaustion resulted in a significant increase in NK cells in the peripheral blood along with increased NK cell lytic activity immediately after exercise (Brahmi *et al.*, 1985). However, NK cell activity was biphasic in that there was a peak immediately following exercise, but 2 hours later the activity was below baseline. Twenty hours after exercise NK activity had returned to pre-exercise levels (Brahmi *et al.*, 1985). These changes may be due to the increased cortisol levels which peak immediately following exhaustive exercise. Interestingly, there was not a difference between the NK cell response to exercise between trained and untrained subjects (training was defined by VO_{2max} and activity levels).

Of significant interest to athletes is whether acute bouts of exhaustive exercise lead to increased infection. Green *et al.* (1981) concluded that long distance running had no effect on immune function since 20 marathon runners had normal blood immunoglobulin values and near normal lymphocyte numbers. However, only 6 of the 20 runners had completed a marathon within 3 days of testing, though 3 had completed a 10-mile run within an hour of blood sampling. Further, only peripheral blood cells from selected subjects were subjected to functional assays. Since the effects of exercise on immune function has been shown to be transient, this study was poorly controlled. Peters & Bateman (1983) reported in an epidemiological study that marathon runners were more susceptible to upper respiratory tract infections within 2 weeks after a race than a nonracing control population. Tomasi *et al.* (1982) suggested that this susceptibility may be due to changes in mucosal immunity immediately after a race since they found that Nordic skiers had decreased levels of salivary IgA before a race and even lower levels afterward. Alternatively, infections in the incubation stage may be aggravated by exhaustive exercise. Reyes & Lerner (1976) reported that swimming to exhaustion caused increased myocarditis of weanling mice infected with coxsackievirus B-3.

Chronic Exercise and Immune Function

Significantly less is known about the effects of training on immune function. Various types of chronic stress have been associated with increased susceptibility to infection due to suppressed immune function (Wing *et al.*, 1988; McIrvine *et al.*, 1982; DePasquale-Jardieu and Fraker, 1979; Keller *et al.*, 1981). However, exercise training differs from stresses such as malnutrition, thermal injury, and trauma in that though exersise is regular, it is not constant. Therefore, it is improbable that regular exercise (training) has the same immunosuppressive effects as chronic physiological stresses. This may be particularly true for those who exercise moderately to maintain cardiovascular fitness since this can be achieved by exercising aerobically 3-4 times per week at a submaximal work load (at least 60% of VO_{2max}) for a minimum of 20 minutes per exercise bout.

Mice trained by treadmill running for 6 weeks had a decreased splenic response to concanavalin A *in vitro* compared to control mice which were exposed to the running environment but not forced to run (Hoffman-Goetz *et al.*, 1986). Interestingly, there was also a significant (25%) reduction in the cellularity of the spleen of trained mice compared to controls. Unfortunately, plasma corticosterone levels were not measured in this study so it is unknown what role glucocorticoids may have played in the decreased lymphocyte function. Ferry *et al.* (1991) reported that after 4 weeks of treadmill training rat thymus and spleen cellularity was not different from that of controls; but, after a fifth week of more intensive training, there was an approximate 20% decrease in the number of cells of both the spleen and thymus. The number of T helper cells decreased in the spleens of trained rats while T cytotoxic/suppressor cells did not change (Ferry *et al.*, 1991). Pahlavani *et al.* (1988) reported that 4 weeks of swim training in rats resulted in a decreased *in vitro* response to concanavalin A but not to lipopolysaccharide. This decrease was age-related since mitogen stimulated proliferation and IL-2 production was reported depressed in 7-month-old rats but not in 18- and 24-month-old rats (Pahlavani *et al.*, 1988). However, a major problem with this study was the failure to show that the rats had achieved a training effect which may have been important in explaining the differences between the different aged rats. Eight weeks of a more moderate training protocol was shown to caused an increased splenic response to con A (Tharp & Preuss, 1991); however, no training effect was shown in this study either.

Moderate exercise training (60% VO_{2max}, 45 minutes/day, 5 days/week) for 15 weeks in humans, involving a walking program for the moderately obese, resulted in significantly higher NK cell lytic activity 6 and 15 weeks after the onset of training and decreased the number of days the subjects reported symptomes of upper respiratory infections (Niemann *et al.*, 1990). Interestingly, this same training program resulted in a slight decrease in the number of peripheral blood lymphocytes (particularly B-cells) after 6 weeks, but they had recovered by 15 weeks of training (Nehlsen-Cannarella *et al.*, 1991). There was also a small increase in serum IgG, IgA, and IgM at 6 and 15 weeks of training. A more rigorous training program in which healthy subjects were exercised for 15 weeks at 70-85% VO_{2max} (50 minutes/day, 5 days/week) resulted in a significant decrease in NK cell lytic activity (Watson *et al.*, 1986). The proportion of peripheral blood T-cells increased with training while the proportion of monocytes decreased. The T-cell response to mitogen stimulation increased with training which may have been related to the increased proportion of T-cells in the peripheral blood. It is obvious from the literature that the contradictions in results are due to the number of different exercise and training protocols which are used. However, generally speaking, moderate exercise and training appears to have an enhancing effect on immune function while overtraining or exhaustive exercise has a more detrimental effect. Unfortunately, many of these studies used *in vitro* functional assays to assess immune response to a number of challenges; and while they provide useful information, it is unknown if they reflect the true state of host defense *in vivo*. Though some animal studies have indicated that exercise does effect spleen and thymus lymphocytes, there is no indication if exercise might also effect hematopoiesis in the bone marrow.

Exercise, Hormones, and the Immune System

While some trends regarding exercise and immune function are emerging from the literature, it is altogether unclear what factors may be regulating the interaction between exercise stress and the immune system. Since glucocorticoids have long been known to have a generally immunosuppressive affect and are elevated during prolonged exercise, it is tempting to attribute to them the interactions between exercise and immune function. In fact, glucocorticoids may be important in suppressing immune function during overtraining in which the testosterone:cortisol ration is decreased (Houmard *et al.*, 1990). However, it is quite clear that glucocorticoids cannot possibly be responsible for all of the effects of exercise on immune function since glucocorticoids aren't elevated during moderate exercise. Other possible mediators between exercise and immune function may be catecholamines and neuropeptides such as the endorphins and enkephalins (Weicker & Werle, 1991). These hormones are released during exercise and have been shown to effect immune function. IL-1 also has been implicated as a link between exercise and immune function since exercise has been shown to cause increased IL-1 release from macrophages (Cannon & Kluger, 1983; Haahr *et al.*, 1991). IL-1 has a number of systemic effects including causing neutrophilia and lymphopenia (Dinarello, 1988). Interestingly, IL-1 and glucocorticoids form a regulatory feedback loop which also may be important in the interaction between exercise and immune function (Besedovsky *et al.*, 1986). Il-1 has been shown to cause the release of ACTH from the pituitary gland which results in glucocorticoid release from the adrenal medulla (Besedovsky *et al.*, 1986; Dinarello, 1988).

Recommendations

There are very few well-controlled studies addressing the effects of exercise or training on immune function. Human studies are difficult in that they are limited to studying immune system cells from the peripheral blood. For this reason animal studies would be more useful for examining immune system organs such as the thymus, spleen, lymph nodes, etc., but there are very few in the literature. Based on the data presented in this dissertation, it would be interesting to examine the role glucocorticoids may play in altering immune function during exhaustive exercise or over-training. Rats would be a logical choice for study since they are relatively easy to train aerobically. It would be interesting to examine the effects of training at various intensities on the cellularity of the thymus, spleen, lymph nodes, and bone marrow. Monoclonal antibodies to rat immune-cell surface markers and flow cytometry could easily be used to examine the changes in subpopulations of cells. This type of a strategy was used in Chapters 2, 3, and 6 of this dissertation. Rats could also be challenged with various antigen *in vivo* and functional studies done. To determine what the role stress hormones play in regulating immune function during exercise training, rats could be adrenalectomized prior to the initation of the training regimens. It would be critical in these studies to carefully monitor the training effect achieved so that comparisons could be made to the work of other investigators.

Though there has been a lot of interest over the last 5 years regarding the effects of exercise on the immune system, there is still much that can be done. However, well-controlled studies need to be performed. This may necessitate the collaboration between immunologists and exercise physiologists since it is uncommon for the same investigator to have expertise in both fields of research.

REFERENCES

Besedovsky, H., Del Rey, A., Sorkin, E., Dinarello, C.A. (1986) Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* 233, 652-654.

Brahmi, Z., Thomas, J.E., Park, M., Park, M., Dowdeswell, I.R.G. (1985) The effect of acute exercise on natural killer-cell activity of trained and sedentary human subjects. *J. Clin. Immunol.* 5(5), 321-328.

Cannon, J.G., Kluger, M.J. (1983) Endogenous pyrogen activity in human plasma after exercise. *Science* 220, 617-619.

DePasquale-Jardieu, P., Fraker, P.J. (1979) The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. *J. Nutr.* 109(11), 1847-1855.

Dinarello, C.A. (1988) Biology of interleukin 1. *FASEB J.* 2, 108-115

Edwards, A.J., Bacon, T.H., Elms, C.A., Verardi, R., Felder, M., Knight, S.C. (1984) Changes in populations of lymphoid cells in human peripheral blood following physical exercise. *Clin. Exp. Immunol.* 58, 420-427.

Eskola, J., Ruuskanen, O., Soppi, E., Viljanen, M.K., Jarvinen, M., Toivenen, H., Kouvalainen, K. (1978) Effect of sport stress on lymhocyte transformation and antibody formation. *Clin. Exp. Immunol.* 32, 339-345.

Ferry, A., Rieu, P., Lasiri, F., Guezennec, C.Y., Abdellah, E., Le Page, C., Rieu, M. (1991) Immunomodulations of thymocytes and splenocytes in trained rats. *J. Appl. Physiol.* 71(3), 815-820.

Fitzgerald, L. (1988) Exercise and the immune system. *Immunol. Today* 9(11), 337-339.

Fitzgerald, L. (1991) Overtraining increases the susceptibility to infection. *Int. J. Sports Med.* 12, S5-S8.

Green, R.L., Kaplan, S.S., Rabin, B.S., Stanitski, C.L., Zdziarski, U. (1981) Immune function in marathon runners. *Ann. Allergy* 47(2), 73-75.

Haahr, P.M., Pedersen, B.K., Fomsgaard, A., Tvede, N., Diamant, M., Klarlund, K., Halkjaer-Kristensen, J., Bendtzen, K. (1991) Effect of physical exercise on in vitro production of interleukin 1, interleukin 6, tumour necrosis factor-α, interleukin 2 and interferon-γ. *Int. J. Sports Med.* 12(2), 223-227.

Hansen, J-B., Wilsgard, L., Osterud, B. (1991) Biphasic changes in leukocytes induced by strenuous exercise. *Eur. J. Appl. Physiol.* 62, 157-161.

Hedfors, E., Holm, G., Ohnell, B. (1976) Variations of blood lymphocytes during work studied by cell surface markers, DNA synthesis and cytotoxicity. *Clin. Exp. Immunol.* 24, 328-335.

Hoffman-Goetz, L., Keir, R., Thorne, R., Houston, M.E., Young, C. (1986) Chronic exercise stress in mice depresses splenic T lymphocyte mitogenesis in vitro. *Clin. Exp. Immunol.* 66, 551-557.

Houmard, J.A., Costill, D.L., Mitchell, J.B., Park, S.H., Fink, W.J., Burns, J.M. (1990) Testosterone, cortisol, and creatine kinase levels in male distance runners during reduced training. *Int. J. Sports Med.* 11, 41-45.

Kappel, M., Tvede, N., Galbo, H., Haahr, P.M., Kjaer, M., Linstow, M., Klarlund, K., Pedersen, B.K. (1991) Evidence that the effect of physical exercise on NK cell activity is mediated by epinephrine. *J. Appl. Physiol.* 70(6), 2530-2534.

Keller, S.E., Weiss, J.M., Schleifer, S.J., Miller, N.E., Stein, M. (1981) Suppression of immunity by stress: Effect of a graded series of stressors on lymphocyte stimulation in the rat. *Science* 213, 1397-1400.

MacNeil, B., Hoffman-Goetz, L., Kendall, A., Houston, M., Arumugam, Y. (1991) Lymphocyte proliferation responses after exercise in men: fitness, intensity, and duration effects. *J. Appl. Physiol.* 70(1), 179-185.

McIrvine, A.J., O'Mahony, J.B., Saporoschetz, I., Mannick, J.A. (1982) Depressed immune response in burn patients. Use of monoclonal antibodies and functional assays to define the role of suppressor cells. *Ann. Surg.* 196, 297-304.

Moorthy, A., Vishnu, Zimmerman, S.W. (1978) Human leukocyte response to an endurance race. *Eur. J. Appl. Physiol.* 38, 271-276.

Nehlsen-Cannerella, S.L., Nieman, D.C., Balk-Lamberton, A.J., Markoff, P.A., Chritton, D.B.W., Gusewitch, G., Lee, J.W. (1991) The effects of moderate exercise training on immune response. *Med. Sci. Sports Exerc.* 23(1), 64-70.

Nehlsen-Cannarella, S.L., Nieman, D.C., Jessen J., Chang, L., Gusewitch, G., Blix, G.G., Ashley, E. (1991) The effects of acute moderate exercise on lymphocyte function and serum immunoglobulin levels. *Int. J. Sports Med.* 12(4), 391-398.

Nieman, D.C., Berk, L.S., Simpson-Westerberg, M., Arabatzis, K., Youngberg, S., Tan, S.A., Lee, J.W., Eby, W.C. (1989) Effects of long-endurance running on immune system parameters and lymphocyte function in experienced marathoners. *Int. J. Sports Med.* 10(5), 317-323.

Nieman, D.C., Nehlsen-Cannarella, S.L., Donohue, K.M., Chritton, D.B.W., Haddock, B.L., Stout, R.W., Lee, J.W. (1991) The effects of acute moderate exercise on leukocyte and lymphocyte subpopulations. *Med. Sci. Sports Exerc.* 23(5), 578-585.

Pahlavani, M.A., Cheung, T.H., Chesky, J.A., Richardson, A. (1988) Influence of exercise on the immune function of rats of various ages. *J. Appl. Physiol.* 64(5), 1997-2001.

Pedersen, B.K. (1991) Influence of physical activity on the cellular immune system: Mechanisms of action. *Int. J. Sports Med.* 12, S23-S29.

Peters, E.M., Bateman, E.D. (1983) Ultramarathon running and upper respiratory tract infections. An epidemiological survey. *S. Afr. Med. J.* 64, 582-584.

Reyes, M.P., Lerner, A.M. (1976) Interferon and neutralizing antibody in sera of exercised mice with coxsackievirus B-3 myocarditis *Proc. Soc. Exp. Biol. Med.* 151, 333-338.

Tharp, G.D., Preuss, T.L. (1991) Mitogenic response of T-lymphocytes to exercise training and stress *J. Appl. Physiol.* 70(6), 2535-2538.

Tomasi, T.B., Trudeau, F.B., Czerwinski, D., Erredge, S. (1982) Immune parameters in athletes before and after strenuous exercise *J. Clin. Immunol.* 2(3), 173-178.

Watson, R.R., Moriguchi, S., Jackson, J.C., Werner, L., Wilmore, J.H., Freund, B.J. (1986) Modification of cellular immune functions in humans by endurance exercise training during β-adrenergic blockade with atenolol or propranolol. *Med. Sci. Sports Exerc.* 18(1), 95-100.

Weicker, H., Werle, E. (1991) Interaction between hormones and the immune system *Int. J. Sports Med.* 12, S30-S37.

Wing, E.J., Magee, D.M., Barczynski, L.K. (1988) Acute starvation in mice reduces the number of T cells and suppresses the development of T-cell-mediated immunity *Immunology* 63, 677-682.