THESIS

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 01564 4655

This is to certify that the

dissertation entitled

PERTURBATION OF LYMPHOPOIESIS BY DIETARY ZINC DEFICIENCY IN YOUNG ADULT A/J MICE

presented by

Farzaneh Osati-Ashtiani

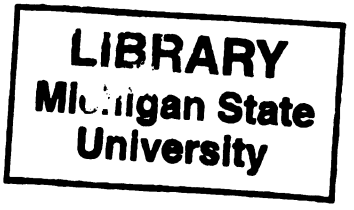
has been accepted towards fulfillment of the requirements for

Ph.D. degree in Pathology

Pamela J. Fraker
Major professor

Date 12/19/96

MSU is an Affirmative Action/Equal Opportunity Institution O-12771



PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.

DATE DUE	DATE DUE	DATE DUE

MSU is An Affirmative Action/Equal Opportunity Institution
c:\circ\circ\datedue.pm3-p.1

PERTURBATION OF LYMPHOPOIESIS BY DIETARY ZINC DEFICIENCY IN YOUNG ADULT A/J MICE

By

Farzaneh Osati-Ashtiani

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Pathology

1996

ABSTRACT

PERTURBATION OF LYMPHOPOIESIS BY DIETARY ZINC DEFICIENCY IN YOUNG ADULT A/J MICE

By

Farzaneh Osati-Ashtiani

Deficiencies in dietary zinc represent a world wide nutritional problem which compromises the host immune defense capabilities, and leads to increased susceptibility to disease and infection. Dietary zinc deprivation in mice, a compatible model for human zinc deficiency, causes rapid thymic atrophy and decreases cell and antibody mediated responses all of which correlates with the losses in the number of peripheral lymphocytes. This nutritional deficiency also results in chronic elevation of endogenous glucocorticoid concentration, which itself is immunosuppressant. Thus, disruption of lymphopoietic processes which could lead to lymphopenia and diminished host defense capacity during zinc deficiency was hypothesized. The primary objective of this research was to evaluate the effects of suboptimal dietary zinc intake on developing B-cells, particularly progenitor and precursor compartments of the B-lineage in the marrow of young adult mice. This investigation was also extended to the examination of the status of T-cells at different stages

of development in the thymus of dietary zinc mice, as the second objective of this dissertation. Since glucocorticoids are known to suppress the immune system, the third objective of this research was to correlate glucocorticoid elevation during zinc deficiency with the suppression of lymphopoiesis through apoptotic cell death of susceptible lymphocytes. The results strongly indicate a selective and stage specific alteration in B-lymphopoiesis in the marrow of zinc deficient mice. Precursor ($B220^+CD43^-IgM^-$) and immature ($B220^+IgM^+IgD^-$) B-cells exhibited significant sensitivity to the effects of zinc deficiency, whereas progenitor ($B220^+CD43^+IgM^-$) and mature ($B220^+IgM^+IgD^+$) B-cells were substantially resistant. Similarly, significant depletion of immature thymocytes ($CD4^+CD8^+$) with concomitant resistance of progenitor ($CD4^-CD8^-$) and mature T-cells ($CD4^+CD8^-/CD4^-CD8^+$) in the thymus indicated a stage specific sensitivity of residual thymocytes to the effects of zinc deficiency. Thus, disruption of lymphopoiesis particularly of early developing immature lymphocytes by zinc deficiency was indeed the underlying cause of impaired immunity. To investigate the role of glucocorticoids in the observed immunological alterations in zinc deprivation, adrenalectomies were performed. Removal of corticosterone via adrenalectomy completely protected thymus integrity and bone marrow cellularity while thymus weights and phenotypic distribution of early developing and immature B-cells were analogous to those found in the control groups. Furthermore *in vivo* and *in vitro* verification of apoptosis in the thymus of zinc deficient mice indicated that the elimination of immature double positive thymocytes ($CD4^+CD8^+$) occurred via apoptosis. Taken together, the results strongly indicate the disruption of lymphopoiesis due to dietary zinc deprivation. Furthermore, a potential role for GC-induced suppression of lymphopoiesis and apoptotic elimination of susceptible lymphocytes in zinc deprived mice are suggested.

Dedicated to:

my husband, Eraj Poureslami

my daughters, Ghazaleh and Bahareh

and

my parents, Ali and Pari Ashtiani.

ACKNOWLEDGMENTS

I am truly grateful to my thesis advisor, Dr. Pamela Fraker, for her guidance, encouragements, and support in the past few years. Pam has shown me in many ways how to be a strong, descent, and honest scientist and I believe my experience as her student will be beneficial for my future career. I am very thankful to Dr. Jon Patterson who served as my academic advisor, for his unconditional time, guidance, and support throughout the years. I greatly acknowledge my other committee members Dr. Richard Schwartz, and Dr. Cheryl Swenson for their guidance, advice and encouragement along the way.

I am also indebted to Dr. Louis King for his significant contribution to this project. Without his guidance, thankless teachings in the use of flow cytometer and suggestions I would not have reached this point. Special appreciation goes to the past and present members of our lab, Dr. Deborah Elghanian, Dr. Bill Telford and my buddy Tonya Laakko for their friendship, advice, and encouragement throughout the years. I would also like to express my appreciation to Teresa Vollmer for her technical assistance that was always available for me.

This section of my dissertation would not be complete without acknowledging my parents. Their continuing and unconditional love, support, and encouragement throughout the years that I have been away from home have been and always will be remembered and

appreciated. I would also like to thank my dear brother and his wife and my lovely niece and nephew for their love and support.

Last but not least, I must thank my dear husband, Eraj, and my beautiful, lovely children, Ghazaleh and Bahareh. Without their love, understanding, sacrifice and unconditional support, completion of this journey would not have been possible. For this and countless other things, I will be always indebted to them.

Thanks for everything.

God bless you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin hormone
Adr	adrenalectomized
AE	*Acrodermatitis entereopathica*
ANOVA	analysis of variance
APA	amino peptidase A
APN	amino peptidase N
AV-PE	avidin-phycoerythrin
BL	basal
BM	bone marrow
CHO	Chinese hamster ovary cells
ConA	concanavalin A
CRF	corticotropin-releasing factor
CRIP	cysteine-rich intestinal protein
CS	corticosterone
DAPI	4',6-diamidine-2-phenylindole
Dex	dexamethasone
DN	double negative

DP	double positive
DTAF	dichlorotriazinyl amino fluorescein
DTH	delayed type hypersensitivity reactions
dUTP	deoxyuridil triphosphate
EDTA	ethylenediaminetetracetic acid
FACS	fluorescently activated cell sorter
FBS	fetal bovine serum
FTS	facteur timique serique
FSC	forward scatter
GC	glucocorticoid
GR	glucocorticoid receptor
HBSS	Hank's balanced salt solution
HI	high
HPA	hypothalamus-pituitary-adrenal axis
HRE	hormone responsive element
HSA	heat stable antigen
hsp	heat shock protein
ICP/AES	inductively coupled plasma-atomic emission spectroscopy
IG	immunoglobulin

IL	interleukin
L-CA	leukocyte common antigen
LDL	low-density lipoprotein
LO	low
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
MNPs	mononuclear phagocytic cells
MRE	metal responsive element
MT	metallothionein
MZD	moderately zinc deficient
NaCl	sodium chloride
NaOH	sodium hydroxide
NIST	national institute of standards and technology
NK	natural killer
NLS	nuclear localization signal
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCD	programmed cell death
PCM	protein calorie malnutrition

PD

PD

PFC

PHA

PHS

ppm

pro-

PTP

RA

RB

RD

RH

RZ

SC

SD

SE

SG

SP

PD	prednisolone
PD	protein deficient
PFC	plaque forming cells
PHA	phytohemagglutinin
PHSCs	pluripotent hematopoietic stem cells
ppm	part per million
pro-B	progenitor B cells
PTPase	protein tyrosine phosphatase
PWM	pokeweed mitogen
RAG	recombination activating gene
RBC	red blood cell
RDA	recommended daily allowance
RHD	rheumatic heart disease
RZA	restricted zinc adequate
SCID	severely combined immunodeficient
SD	standard deviation
SERPIN	serine protease inhibitor
SOD	superoxide dismutase
SP	single positive

SRBC	sheep red blood cells
SSC	side scatter
SZD	severely zinc deficient
TCR	T-cell receptor
TD	T-cell dependent
TdT	terminal deoxynucleotidyl transferase
TEC	thymic epithelial cell
TF III A	transcription Factor III A or Factor A
TI	T-cell independent
TNP-LPS	trinitrophenyl conjugated lipopolysaccharide
UN	undetectable
ZA	zinc adequate
ZD	zinc deficiency/zinc deficient

Introduction

The importance of zinc as a micronutrient in the growth and development of mammals was first recognized by Todd *et al.* in 1934. Since then, a growing body of literature has been devoted to the importance of zinc as well as deleterious effects of its deficiencies in biological systems, particularly in immune system (Prasad, 1985; Fraker *et al.*, 1986; 1993; Endre *et al.*, 1990; Walsh *et al.*, 1994; Hadden, 1995; Cousins, 1996). Being one of the largest tissues in the body and a dynamic system with constant development, differentiation, and proliferation, the immune system requires a continuous flow of nutrients including zinc for its proper function. The frequency of zinc deficiency (ZD) in humans and its adverse effects on the immune components emphasizes the necessity for understanding the role of zinc in immunity. The high reliability of mice as an immunological model for humans has greatly enhanced our knowledge about the interrelationship between zinc deficiency and impairment of the immune system. Information gathered to date indicates that a period of 30-day insufficient zinc intake in young adult mice is sufficient to cause: 1) weight loss, 2) rapid thymic atrophy; and 3) reduced numbers in lymphocytes and macrophages in the peripheral immune system (eg., spleen) (Fraker *et al.*, 1986; 1993). Furthermore, the deficiency caused severe depressions in antibody and cell mediated immune responses (DePasquale-Jardieu and Fraker, 1984; Fernandes *et al.*, 1984; Jardieu and Fraker, 1990). Further investigations revealed that in

spite of severe depletion of lymphocytes in the spleen, the proportion of residual splenocytes (T-cells to B-cells) were normal (King *et al.*, 1991). Similarly, the functional capacity of residual splenocytes in terms of their proliferation capacity and antibody and cytokines production in response to different mitogenic stimuli was normal (Cook-Mills and Fraker, 1993a). Taken together, it appeared that lymphopenia was the most probable cause for reduced host defense capacity. Thus, alteration in lymphopoiesis in the bone marrow (BM), the site of lymphopoiesis in adult mammals, appeared to be a possibility causing reduction in lymphocyte numbers and impaired immunity in ZD. This hypothesis was used as the main frame for investigations on this dissertation. The primary objective of this dissertation was to identify the status of B-lymphopoiesis in the marrow and T-cell maturation in the thymus of ZD mice. It was of interest to determine if suboptimal levels of zinc result in a uniform reduction in lymphocyte subsets or if selective alterations occur in certain subclasses. Experiments reported in Chapters two and three will extensively examine different stages of B-cell development through immunophenotyping the marrow of mice on a 27-day zinc deficient dietary regime. Likewise, Chapter five will address the status of T-cell maturation in the thymus of dietary zinc mice, as the second objective of this research.

Chronic elevation of glucocorticoid concentrations (GCs) in the later stages of ZD has been established (Quarterman, 1972; 1974; Quarterman and Humphries, 1979; DePasquale-Jardieu and Fraker, 1979; Prasad, 1985). DePasquale-Jardieu and Fraker demonstrated that ZD constitutes a stress that activates the hypothalamus-pituitary-adrenal cortex axis (stress axis) and subsequently leads to the chronic production of corticosterone (CS, the predominant form of GCs in murine system) (DePasquale-Jardieu and Fraker, 1979). Furthermore,

removal of CS via adrenalectomy protected against involution of the cortex in which immature thymocytes reside (DePasquale-Jardieu and Fraker, 1980). Thus, it appeared that GC had profound effects on thymic integrity, in particular on immature thymocytes, as has been also shown by others (Quarterman and Humphries, 1979; Miller *et al.*, 1991; 1994; Flaherty *et al.*, 1993). Recent literature has extensively demonstrated the sensitivity of immature thymocytes to GCs followed by induction of apoptosis as a mean for the elimination of vulnerable lymphocyte populations (Cohen and Duck, 1992; Sun *et al.*, 1992; Brown *et al.*, 1993; Cidlowski *et al.*, 1996). In fact, several recent studies from this lab have indicated the susceptibility and apoptotic elimination of thymocytes as well as developing B-cells to short exposure of low levels of synthetic (dexamethasone) and natural (corticosterone, cortisol) GCs *in vitro* (Garvy *et al.*, 1991; 1993b; Voetberg *et al.*, 1994). These findings provided a foundation for the two other objectives in this dissertation. Thus, the third objective of this research was to identify the role of endogenously elevated levels of CS in B-lymphopoiesis in the marrow of ZD mice. This objective was met by the removal of GCs from the circulation via adrenalectomy. It was of interest to investigate whether removal of the GC would protect BM cellularity in ZD mice as it did for the thymus (DePasquale-Jardieu and Fraker, 1980). This will be addressed in Chapter Four. The final objective was to verify whether apoptosis played a role in elimination of GC-susceptible lymphocyte populations in ZD mice. Use of a homogenous tissue such as thymus provided an easier tool for this investigation. Chapter Five, along with evaluation of T-cell subsets in ZD, concomitantly examines the presence of apoptosis among immature thymocytes in the thymus of ZD mice. Due to rapid *in vivo* phagocytosis of apoptotic cells by phagocytic cells (Savill *et al.*, 1989a;

1989b; Cohen, 1991; Evan *et al.*, 1992), the verification of apoptosis in ZD was attempted through two approaches. In the first approach (*in vivo* system), freshly isolated thymocytes from different dietary groups were shortly incubated (6 hrs) in regular media (RPMI-1640) to allow for the completion of apoptotic processes in cells that received the death signal *in vivo*, but escaped phagocytosis. In the second approach (*in vitro* system), freshly prepared thymocytes from regular mice were incubated (8 hrs) in culture media (RPMI-1640) in which known levels of CS and zinc relevant to the levels detected in zinc dietary mice were supplemented. This approach allowed for the identification of the role of zinc and CS or their synergistic action in thymocyte survival or death and allowed detection of the apoptotic elimination in immature thymocytes with higher magnitude compared to the *in vivo* system. Identification of apoptotic cells were made possible by a rapid and highly quantitatively method recently introduced by this lab (Telford *et al.*, 1991). Using multicolor FACS analysis for the identification of different subpopulations along with their simultaneous cell cycle analysis via fluorescent DNA dye, the precise proportion of each subpopulation undergoing apoptosis was rapidly detected.

Collectively, these studies strongly indicate a selective disruption in lymphopoietic processes in zinc deprived mice. Further, they indicate a potential role for GC in alteration in lymphopoiesis leading to apoptotic elimination of susceptible lymphocytes.

CHAPTER ONE

LITERATURE REVIEW

SECTION I: Biochemical and Physiological Significance of Zinc

Introduction: It was not until 1934 that zinc was shown to be necessary for the growth of mammals (Todd *et al.*, 1934). Later in 1963, the importance of zinc for human health was first documented by Prasad and his colleagues. This documentation was based on clinical studies on patients from Iran (Prasad *et al.*, 1961) and Egypt (Prasad *et al.*, 1963) who demonstrated severe growth retardation, hypogonadism, hepatosplenomegaly, rough skin, geophagia (in male Iranian subjects) and *Schistosomiasis* (in Egyptian patients). In addition the zinc content of plasma, red blood cells and hair was dramatically decreased in dwarfs compared to normal subjects. The diet of these patients consisted of bread made of wheat flour which is known to contain high phytate, which decreases the availability of zinc. These clinical manifestations and laboratory observations indicated zinc deficiency as a principal feature in these patients. Since then investigations related to metabolic functions and essentiality of zinc has expanded rapidly. The essential role of zinc in the survival, growth and integrity of cellular components of different organisms is due in part to the utilization of this element by more than 200 metalloenzymes for their function (Coleman, 1992; Vallee and Auld, 1990). In these enzymes, zinc participates in catalytic, cocatalytic (coactive) or structural activities (Vallee and Falchuk, 1993). Thus, if the metal is removed by chelating agents or if it is not available at optimal levels (eg., dietary zinc deficiency) the catalytic functions and structural stability of these zinc-dependent enzymes will be abolished, thereby leading to profound physiological alterations. The biochemical features of zinc have lead to the recognition of zinc in wide variety of biological systems. In the last few decades many investigators have specified the significant role of zinc in biomembrane integrity, wound healing, reproduction, the nervous system, carcinogenesis, the endocrine system, and as an

antioxidant and therapeutic agent (Prasad, 1988; Shambaugh, 1989; Endre *et al.*, 1990; Watanabe *et al.*, 1995). However, this chapter will review the characteristics and the importance of zinc in biological systems and the impact of its deficiencies on some components of the immune system.

Chemical Properties: Zinc is widely known as one of the most ubiquitous elements in the nature and one of the most essential trace metals in the human body. This element with an atomic number of 30 and atomic weight of 65.37 is placed in Group II B transition elements in the periodic table (Williams, 1989; Walsh *et al.*, 1994). Zinc is generally present as a divalent cation (Zn^{2+}) with a completely filled d shell (10 d electrons) which allows zinc to play its unique structural roles. First, it readily undergoes ligand exchange reactions which explains its catalytic role in metalloenzymes. Second, as an electron acceptor molecule, it can strongly interact with ligands such as thiolate from cysteine, amine from histidine, oxygen from aspartate, glutamate and water. Third, it shows no tendency for oxidation and reduction activities which highlights its biological functions, since redox activity results in the free radical formation (Williams, 1989; Da Silva and Williams, 1991). In fact zinc is known to act as free radical scavenger by displacing redox-active transition metals (eg., iron and copper) from their specific site and preventing hydroxyl radical ($OH^{\cdot}$) formation at the active site (Bovering and Dean, 1992; Powell *et al.*, 1990). The scavenging mechanism of zinc was demonstrated in a recent study by Powell *et al.*, (1994) in which they showed that the perfusion of isolated rat hearts with zinc significantly improved the cardiac postischemic recovery. When they measured the copper concentration in hearts treated with zinc (30μM), they noticed 27% less copper than the control hearts. Thus it seems that by competing with

copper and displacing it, zinc could suppress the $OH^{\cdot}$ formation and improve postischemic recovery.

The other mechanisms by which zinc may function as an antioxidant is the protection of sulfhydryl groups against oxidation. This is achieved by direct binding of zinc to sulfhydryl groups, thus preventing intramolecular disulfide formation (Bray and Bettger, 1990). Furthermore, the presence of zinc at the active site of superoxide dismutase (SOD) (eliminates superoxide anions, $O_2^{\cdot}$, formation), and its association with metallothionein (a free radical scavenger) indicates the indirect contribution of zinc in antioxidation activities and intensifies the importance of zinc in biological systems (Bray and Bettger, 1990). In fact a study by Richard *et al.*, (1991) reported high lipid peroxidation and tissue injury in patients with chronic renal failure due to decreased enzymatic activity of SOD. The decrease in SOD activity and subsequent hyperlipoperoxidation in these patients were explained by a disturbance in the status of zinc as decrease in serum zinc level was also noted. SOD converts superoxide to molecular oxygen and hydrogen peroxide which is subsequently degraded to oxygen and water by catalases and proxidases. Thus, by removing these highly reactive radicals via scavenging capacity of SOD, the tissue damage is prevented (Borg *et al.*, 1992).

Taken together, these data suggest that zinc is involved in antioxidative defense systems; thus, its deficiency could result in an increase in tissue oxidative damage. Recent studies from different laboratories have in fact shown the oxidative damage to proteins and lipids in rats fed a low zinc diet due to the low zinc metalloenzymes activities and increased reactive oxygen species (Coudray *et al.*, 1991; Disilvestro and Carlson, 1994; Oteiza *et al.*,

1995). Thus, some of the observed physical and biological alterations in zinc deficient mice studied in this dissertation could be related to the disturbance in the antioxidative mechanisms.

Zinc Metabolism:

Absorption: Zinc commonly enters the body through ingestion of food and drink and its absorption is primarily by the small intestine including duodenum, jejunum and ileum (Solomons and Cousins, 1984; Cousins, 1985; Cousins, 1989; Lönnerdal, 1989). Most recently rat colon was identified as another site of absorption (14%) for this nutrient (Naveh *et al.*, 1993). Although zinc absorption has been investigated widely, the mechanism (s) involved in this process has not been clearly identified. However, most of the investigators think that zinc absorption occurs via diffusion (nonmediated) and carrier-mediated mechanisms (Cousins, 1989; Lönnerdal, 1989). At high zinc concentrations, the brush border membrane of the intestinal mucosal cells absorb zinc by passive diffusion (Tacnet *et al.*, 1990; Raffaniello *et al.*, 1992) and under normal dietary conditions zinc is absorbed by the carrier-mediated mechanism which is most active at low luminal zinc concentration (Raffaniello and Wapnir, 1989; Hempe and Cousins, 1991). Thus, one would expect that at suboptimal dietary zinc intake the intestinal mucosa would have higher zinc uptake. In fact in an earlier study by Steel and Cousins (1985) they demonstrated that the intestinal absorption rate in the zinc deficient rats was eight fold higher than in the control rats, suggesting an increase in membrane transport mechanism when the zinc supply decreases. However, there was no indication of whether or not the higher zinc uptake in these patients was associated with zinc mobilization from other tissues (eg., muscles, bone) which can affect the tissue zinc distribution. This will be addressed in Chapter Three of this dissertation.

The carrier-mediated zinc absorption is regulated via two proteins, namely, metallothionein (MT) and cysteine-rich intestinal protein (CRIP) (Raffaniello and Wapnir, 1991; Hempe and Cousins, 1992). This mechanism was primarily identified as a low molecular weight (6500 daltons) cysteine rich protein, namely metallothionein (MT) (Hoadley and Cousins, 1987; Seal and Heaten, 1987). This protein which binds many divalent cations (eg., Zn, Cu, Cd, etc.) has been suggested as a storage protein for zinc (Chesters, 1991). MT synthesis is directly correlated to dietary zinc intake and inversely related to zinc absorption (Hoadley *et al.*, 1988; Cousins and Lee-Ambrose, 1992). The interrelationship between zinc and MT and its relevancy to this dissertation will be addressed in this section.

The cysteine-rich intestinal protein (CRIP) has been identified as a 77-amino acid, 8.6 KDa protein with seven cysteine residues (Birkenmeier and Gordon, 1986). This protein is mainly expressed in small and large intestine with minimal to no expression in other tissues (Hempe and Cousins, 1991). A recent study by Hempe and Cousins (1992) was specifically aimed at the identification of a role for CRIP in zinc absorption and the interrelationship between CRIP and MT in the regulation of zinc transport. Using isolated intestinal loops from rats fed either low (1 μg zinc/g) or high (180 μg zinc/g) zinc diet, an inverse relationship in zinc absorption was shown between these two proteins suggesting a competitive binding interactions between MT and CRIP for intracellular zinc transport.

Many agents affect zinc absorption. Agents that form insoluble complexes with zinc are the primary source of malabsorption. For example, phytate (a phosphate rich compound found in cereal grains, legumes and soy-based infant formulas) histidine and cysteine (high affinity binding amino acids), have all been shown to reduce or inhibit zinc absorption (Han

et al., 1994; Cousins, 1996). Thus, due to high affinity binding of zinc to many ligands (eg., phosphate groups, sulfhydryl groups) and the possible formation of insoluble complexes with zinc, malabsorption of zinc with a subsequent zinc deficiency is expected. In fact, human zinc deficiency in the Middle East due to the high consumption of plant proteins which contain large amounts of phytate (inositol hexaphosphate) is a classical example of zinc malabsorption and zinc deficiency (Prasad, 1963; Prasad, 1991). Furthermore, clinical problems of gastrointestinal absorption, kidney disorders, alcohol, and inborn errors of metabolism also contribute to the zinc malabsorption (Cousins, 1985; Walsh *et al.*, 1994). This dissertation, however, will specifically address the adverse effects of suboptimal dietary zinc intake and its outcomes on some immune components of the murine system.

Transport: Plasma is the major route of zinc transport throughout the body once it has been absorbed. The total zinc content of plasma varies from 95-130 mg/dl in a healthy adult. However, it comprises only 0.1% of the total body zinc (Cousins, 1989; Bremner and May, 1989). Within the plasma, zinc appears to be bound to two main carrier proteins, namely macromolecular and micromolecular zinc ligands. The macromolecular zinc ligands which comprise more than 98% of the circulating zinc include transferrin, albumin and α_2-macroglobulin (Cousins, 1989; Walsh *et al.*, 1994; Cousins, 1996). Approximately 66% of the plasma zinc is bound to albumin presumably to one of the histidine moieties of this molecule. Albumin is considered as the major and the more dynamic source of zinc binding protein and zinc carrier within the circulation. Approximately one-third of the zinc carried in the plasma is bound to α_2-macroglobulin which comprises a tightly bound pool of plasma zinc. The incorporation and metabolism of this complex only occurs in the liver. The physiological

significance of zinc binding α_2-macroglobulin has not been identified yet. The micromolecular portion of the zinc binding carriers which comprise only 1-2% of the circulating zinc is composed of amino acids such as histidine, cysteine, glutamine, and threonine. The amino acid-zinc complexes, due to their low molecular weights, are able to transport to all tissues and body organs including brain. This small zinc pool may act as a zinc donor for a high affinity zinc uptake system within cells (Cousins, 1989; Cousins, 1996).

Distribution: Zinc is found in almost all organs, tissues and body fluids, however, at different concentrations. The highest tissue concentrations of zinc have been found in human prostate gland (520 mg/kg dry weight), muscle (197-226 mg/kg dry weight), bone (218 mg/kg dry weight), kidney (184-230 mg/kg dry weight), liver (141-205 mg/kg dry weight), and pancreas (115-135 mg/kg dry weight) (Walsh *et al.*, 1994). The rest of the tissues contain relatively trace amounts of zinc. The zinc content in extracellular fluids is relatively low (plasma contains only 0.1% of the total body zinc) compared to intracellular stores (about 99%) (Vallee and Falchuk, 1993). However, plasma zinc serves as a primary source of the element available to all cells. Thus, small variations in the zinc content of tissues would have dramatic effects on plasma zinc. Furthermore, any fluctuations in the dietary zinc intake could have the same consequences. A decrease in plasma zinc levels has been repeatedly reported in both humans and animals following zinc deprivation where introduction of a zinc deficient diet was followed by rapid depression of the plasma zinc (Endre *et al.*, 1990; Prasad, 1991; Cook-Mills and Fraker, 1993a; King *et al.*, 1995).

The distribution of zinc between extracellular fluid pool and intracellular pools has been shown to be sensitive to several factors. These include variations in hormonal balance

(eg., glucagon, insulin and glucocorticoids), inherited disorders of zinc metabolism(eg., *Acrodermatitis enteropathica*) and diets either low in zinc or with zinc chelating agents, in all of which the plasma zinc level is depressed (Cousins, 1985; Bunce, 1989; Jackson, 1989). This is of significant interest to this dissertation where a combination of zinc deprivation and chronic elevation of glucocorticoids which accompanies zinc deficiency could be evaluated for their role in zinc mobilization. This subject will be addressed in Chapter Three of this dissertation.

Excretion: Following zinc absorption, the unabsorbed portion of the ingested zinc which is in the form of insoluble complexes is carried in the intestine and excreted in the feces. Thus fecal zinc is composed in large part of unabsorbed zinc. A portion of the zinc in feces also reflects the secreted zinc from gastrointestinal tract and the bile. Zinc in feces represents the major source of zinc lost from the body (70-80% of the total ingested zinc). Fecal zinc excretion ranges from 5-10 mg/day and depends on the dietary zinc intake and physiological condition (Cousins, 1985; Jackson, 1989).

A small portion of zinc is also excreted in the urine of healthy subjects. This portion is composed of zinc primarily bound to amino acids. The zinc-amino acid complexes would then readily pass the renal glomerulus and are excreted in the urine. The urinary zinc excretion (approximately 500-800 μg/day) reflects changes in nutrition, disease and physiology of both humans and animals. For example, urinary zinc loss is a common feature of acute and chronic liver disease and renal disease in which, due to the damage to the cells and their function, zinc can not be handled properly and is excreted in the urine(Barry *et al.*, 1990). A small amount of zinc is also excreted in sweat (115 μg/dl) which under extreme heat would increase to as

high as 2-3 mg daily. There is no known body store for zinc with the exception of zinc stored in the bone. Therefore, homeostasis of zinc mostly depends on the balance between absorption and excretion where an adequate nutritional diet is provided.

Role of Zinc in Metalloenzymes: The essential role of zinc in metabolism, transmission of genetic messages, growth and development is mainly due to association of zinc as an integral component of over 200 zinc metalloenzymes (Walsh *et al.*, 1994; Coleman, 1992). By incorporation of zinc into enzymes such as carbonic anhydrase, which dehydrates bicarbonate in the lungs and hydrates carbon dioxide from other tissues; alcohol dehydrogenases that catalyze the oxidation of alcohols and certain steroids; carboxypeptidases which catalyze carboxyl-terminal peptide bond hydrolysis in peptides and proteins; alkaline phosphatases that catalyze the hydrolysis of a variety of phosphate esters; nucleotide polymerases which are involved in DNA and RNA synthesis; superoxide dismutase that catalyzes the production of hydrogen peroxide; and many other zinc-dependent enzymes, the structural stability and the biological activities of these enzymes are maintained (Vallee and Glades, 1984; Vallee and Auld, 1990; Vallee and Auld, 1993).

Carbonic anhydrase is an enzyme in which the catalytic activity could be impaired by insufficient zinc. In patients with sickle-cell disease in which a conditioned zinc-deficient state is observed, the content of carbonic anhydrase in the erythrocytes was decreased in correlation with the zinc content of the erythrocytes (Vallee and Auld, 1990).

Another interesting case is the liver alcohol dehydrogenase in which both catalytic and structural roles of the zinc ion can be found. This enzyme has two active sites and four ions of zinc per molecule. Two zinc ions are essential for catalytic activity and are bound to the

enzyme via two cysteinyl-SH groups and the imidazole ring. The other two zinc ions appear to have a structural function and are each bound to four cysteinyl-SH groups (Vallee and Auld, 1990).

In some cases zinc plays a non-catalytic role in the zinc-dependent enzymes. For instance, in case of superoxide dismutase, There are two atoms of copper and two atoms of zinc present per molecule of protein and all four atoms are required for optimal superoxide dismutase activity. Zinc is thought to be needed for the stability of the enzyme (Bremner and Beattie, 1990). This enzyme which is a member of oxidoreductases, zinc metalloenzymes, catalyzes the production of hydrogen peroxide in aerobic cells which in turn protect the cells from the oxygen free radicals formation. The protective activity of SOD against free radical formation could be either through dismutation of superoxide anion (O_2^-) or by preventing iron (Fe^{2+}) reacting with hydrogen peroxide, thereby generating free hydroxyl radical ($OH^{\cdot}$) in the well-known Fenton reaction (Willson, 1989; Bray and Bettger, 1990; Bremner and Beattie, 1990; Borg *et al.*, 1992).

In addition to the cytosolic copper and zinc containing SOD (Cu-ZnSOD), a mitochondrial manganese-containing SOD (MnSOD) has been also identified in mammalian tissues (Borg *et al.*, 1992; Giglio *et al.*, 1994). The effects of zinc deficiency on antioxidant activities of Cu-ZnSOD and MnSOD was investigated by Taylor and colleagues (1988). The Cu-ZnSOD activity was shown to be unchanged in the liver and elevated in the lungs of the zinc deficient rats. The MnSOD activity were unchanged in the liver and lung of zinc deficient rats compared to zinc adequate controls. In general, even though the animals were severely zinc deficient, the changes in the free radical defense system were small. Thus, it

seems that the antioxidant defense system may be so important to the survival of the zinc deficient animal that the growth of the animal is halted in order to maintain tissue zinc concentrations and other components of the antioxidant defense mechanism.

Furthermore, zinc has been recognized as a structural regulator of many transcription factors that are necessary for gene activation. The structural role for zinc in transcription factors was first recognized in transcription factor IIIA (TFIIIA) from frog *Xenopus leavis* (Miller *et al.*, 1985). Most of these transcription factors including TFIIIA include small zinc-based domains called zinc fingers that are necessary for DNA recognition and subsequent gene transcription (Rhodes and Klug, 1993). Over the past decade, more than ten classes of such zinc finger domains have been discovered and characterized (Schwabe and Klug, 1994). This subject will be discussed in more detail in upcoming section under zinc and gene transcription.

Metallothionein: Metallothionein (MT) is a significant macromolecular ligand for zinc with a unique structural features including low molecular weight (<10,000 daltons), a high content of heavy metals (4-12 atoms/mole) and high cysteine content (30% of the residues). Mammalian MT is a 61-62 amino acid peptide containing 20 cysteines, 6-8 lysines, 7-10 serines, one acetylated methionine at the amino terminus and no aromatic and hydrophobic amino acids. The majority of cysteine residues are present in cys-x-cys and cys-cys sequences where x represents any other amino acid (Hamer, 1986; Bremner and Beattie, 1990; Cousins, 1985; Chesters, 1992). MT binds the essential metals copper and zinc under physiological conditions and the toxic metals cadmium and mercury under pathological conditions (Nagel and Vallee, 1995). This protein (MT) has higher binding affinity for copper

relative to zinc, whereas its induction is more responsive to dietary zinc than to copper (Cousins, 1985). In fact, the low stability constant of zinc-MT would serve as labile source of zinc that could be utilized for the activation of many zinc-dependent enzymes. All the cysteine residues are involved in metal binding with some of the cysteine residues sharing the metal ion. Metals are associated with MT through thiolate bonds to all 20 cysteine residues (tetrahedrally coordinated to four cysteine thiolate ligands) and are contained in two distinct clusters. The A cluster contains eleven cysteines, binds four atoms of zinc or cadmium or five to six atoms of copper, and is contained within the carboxyl-terminal α domain. The B cluster contains nine cysteines, binds three atoms of zinc or cadmium or six atoms of copper and is contained in the amino-terminal β domain(Hamer, 1986; Vallee and Auld, 1990; Kay *et al.*, 1991).

MT has been isolated from a variety of species and a wide range of tissues, including liver, kidney, pancreas, intestine, brain, thymus, bone marrow and reproductive organs. However, the concentration of the protein in tissues is highly variable and is influenced by many nutritional (dietary), physiologic and developmental factors (Bremner, 1987; Bremner and Beattie, 1990; Huckle *et al.*, 1993). For example, the concentration of MT is greatly reduced in tissues of zinc-deficient animals (Bremner *et al.*, 1987; Vruwink *et al.*, 1988; Cousins and Lee-Ambrose, 1992) and are increased after induction of many types of stress or metal administration (Cousins, 1985). Its ubiquity, and inducibility by a wide range of stimuli, including zinc, copper, infections, and stress suggest that it plays a vital role in the regulation of metabolic processes that utilize this essential trace element.

It has been shown that MT performs an important regulatory role in the cells by being involved in the intracellular phase of zinc absorption, in cellular detoxification or storage of zinc, and in direct activation of zinc dependent enzymes by donating its metal to apoenzymes and converting them into fully active enzymes (Hamer, 1986; Bremner, 1987; Vallee and Falchuk, 1993). *In vivo* and *in vitro* studies have suggested a role as a free radical scavenger as it has been shown to be a powerful binder of hydroxyl radicals (Thornally and Vasak, 1985; Bremner and Beattie, 1990; Schwarz, 1994; Powell *et al.*, 1994; Palmiter, 1994). Furthermore, rapid induction of MT synthesis in response to increased intracellular zinc concentrations is consistent with its action as a scavenger of metal ions. Thus in zinc deficiency where zinc is at suboptimal levels, MT synthesis would be diminished, thereby the removal of toxic heavy metals such as cadmium and to some extent copper will be impaired. This could result in free radical formation, lipid peroxidations, and subsequent tissue damage.

Synthesis: The Synthesis of the major storage-regulating metalloprotein (metallothionein) is triggered by increasing levels of the free metal ions (eg., Zn,Cd,Cu) in the cell (Hamer, 1986; Palmiter, 1994). In the liver, metallothionein synthesis functions in uptake and storage of zinc in hepatocytes. In the intestinal mucosal cells, this protein competes with CRIP,a zinc-binding ligand involved in zinc absorption, regulating the amount of zinc available for transfer to the plasma as discussed (Cousins, 1983; 1989; 1996). Thus, the MT synthesis controlled by the plasma zinc concentration demonstrates a homeostatic mechanism in zinc metabolism.

In an early investigation by Richards and Cousins (1975), they demonstrated zinc regulation of an actinomycin-D sensitive mechanism that involves the synthesis of hepatic and

intestinal metallothionein. Their data clearly showed that plasma zinc uptake by hepatocytes must involve metallothionein synthesis since this is the only zinc binding protein that has the dynamic capacity to respond to changes in zinc status. So they proposed that when plasma zinc levels are high, liver metallothionein synthesis is stimulated which facilitates the uptake of zinc into hepatocytes. Zinc remains bound to this protein until needed to meet cellular requirements. Furthermore, The control of metal-induced MT synthesis at the transcription level has been shown by inhibitory effects of cycloheximide, actinomycin D, and puromycin on the process (Durnam and Palmiter, 1987; Raffaniello and Wapnir, 1991). This effect has been confirmed by measurement of mRNA levels using cell-free translation systems and by direct assay using cDNA probes. The rate of protein synthesis closely parallels the production of metallothionein mRNA (Durnam and Palmiter 1987), and a high rate of transcription can be detected within one hour of stimulation by metals. The mRNA levels reach a maximum of at about 6-8 hours after exposure to an inducer, although the maximal levels of metallothionein occur after 1-2 days (Palmiter, 1987). Cadmium is a particularly potent inducer of metallothionein synthesis (Webb, 1986). The dynamics of metallothionein synthesis in relation to zinc and copper metabolism has been comprehensively reviewed (Cousins, 1985). Generally, a close relationship exists between zinc status and the levels of metallothionein in tissues.

In a recent study by Cousins and Lee-Ambrose (1992), the influence of dietary zinc levels in the regulation of MT gene expression in rats was demonstrated. Utilizing different dietary levels of zinc (5,30,180 mg Zn/kg), they showed that the intestine and liver took up more zinc than other tissues. Nuclei purified from liver, kidney, and spleen accumulated

substantial amounts of zinc which was directly related to the amount of dietary zinc intake. Furthermore, northern analysis demonstrated that MT expression was also proportional to dietary zinc intake, where it was greatest in kidney followed by liver, intestine, spleen and heart. These data strongly suggest that the induction of MT synthesis is directly correlated with dietary zinc supply as well as the bioavailability of zinc. Although not specifically addressed in this dissertation, the possibility of reduced MT synthesis due to suboptimal zinc intake should be considered as one of the underlying causes of observed manifestations of zinc deficiency.

There is also a number of nonmetal factors which induce metallothionein synthesis. Many of the stress inducers which raise circulating levels of glucocorticoids (GCs) stimulate metallothionein synthesis in the liver and to a lesser extent in the heart, kidney, skeletal muscle, and spleen of the mouse (Hager and Palmiter, 1981). Glucocorticoids were therefore thought to mediate some of the effects on MT synthesis. Other steroid hormones, such as estrogens and progesterone, can also induce MT synthesis (Kagi and Schaffer, 1988). Endotoxin is another potent inducer of MT synthesis in the liver and other tissues. This process is mediated by the release of cytokines (De *et al.*, 1990). Rats injected with endotoxin showed reduced serum zinc levels only 3 hrs after treatment and greatly elevated hepatic metallothionein after 18 hrs (Abe *et al.*, 1987). Furthermore, hepatic metallothionein mRNA levels in Syrian hamsters increased fourfold, 6 hrs after administration of endotoxin (Etzel *et al.*, 1982).

In fact, in a recent study, a combination of regulatory factors such as zinc, copper, endotoxin and glucocorticoids on MT synthesis in different tissues, specially brain, was

investigated (Gasull *et al.*, 1994). Administration of zinc, glucocorticoids (dexamethasone, corticosterone) and endotoxins significantly increased the level of MT concentrations in the liver and kidney, with non-uniform distribution of MT in different areas of the brain. When they examined the effect of zinc deficiency on MT synthesis, by feeding rats a zinc deficient diet, they observed lower liver and serum MT and zinc concentrations compared to the control group, with no significant change in the MT levels in different parts of the brain. Once again these data confirm the multi-factor regulation of MT synthesis.

Bacterial infection also induces a marked increase in hepatic metallothionein levels and a decrease in serum zinc concentrations (Sobocinski *et al.*, 1978), both characteristic of acute-phase response. Several mechanisms have been suggested for the induction of metallothionein synthesis during infection. Interleukin-1 (IL-1), which is produced and released from monocytes and activated macrophages in response to infection, stimulates the synthesis of metallothionein and uptake of zinc by the liver of male (Cousins and Leinart, 1988) and pregnant female (Held and Hoekstra, 1984) rats. This finding suggests that GCs mediate some of the cytokine-stimulated induction of metallothionein, since IL-1 causes the release of these hormones via stimulation of adrenocorticotropin hormone (ACTH) release (Held and Hoekstra, 1984). More recently Schroeder and Cousins (1990) showed the effects of IL-6 as a major cytokine mediator of MT gene expression and zinc metabolism in rat hepatocytes. However, bone marrow MT gene expression was shown to be solely dependent on dietary zinc and not on IL-1 or IL-6 (Huber and Cousins, 1993).

Functions of MT: Unique features of the MT include its expression in most tissues, its high content of heavy metals (eg., zinc, copper, cadmium, mercury, etc.) and its being a

highly conserved protein in evolutionary terms. As was mentioned earlier, MT has been shown to act in the detoxification of heavy metals especially cadmium (Webb, 1987), and in the control in zinc and copper homeostasis via regulating their intestinal absorption in different physiological conditions (Cousins, 1985; Menrad *et al.*, 1981; Richards and Cousins, 1976). MT also acts as a metal transfer protein, a metal-storage protein, a sulfur storage protein, an acute phase protein, and a free radical scavenger. The antioxidant activity of MT could be viewed as a mechanism in cells resistance to cancer chemotherapy. Cells with acquired resistance to antineoplastic agents have shown overexpression of MT which tends to bind these alkylating agents to a higher extent than the non-resistant cells (Ebadi and Iverson, 1994). The mechanisms underlying this observation have not been clearly identified. However, the results could be partly explained by the detoxification and free radical scavenging mechanisms of MT. Increased accumulation of MT occurs in the liver and to a lesser extent in bone, thymus and other tissues in animals subjected to different types of stress (Bremner and Beattie, 1990). These stresses include restriction of food intake, bacterial infection, and inflammatory and physical stress such as exposure to high or low temperatures. The increased synthesis of metallothionein is accompanied by increased tissue zinc concentrations, and the incorporation of zinc into the MT structure (Karin and Herschmann, 1981). The hypozincemia that usually occurs in stressed animals appears to be a consequence of the induction of hepatic metallothionein synthesis via increased zinc uptake from the circulation (Cousins, 1989). The influx of zinc into the cells under these circumstances plays key roles in DNA synthesis and gene expression. Moreover, the induction of MT gene

expression and synthesis which provide free-radical scavenging mechanism is another beneficial aspects of increased cellular zinc uptake in stressed subjects.

In zinc deficiency, where a combination of low zinc availability and increased stress hormones (GCs) is present, a more complex phenomenon is expected. It is possible that elevation of glucocorticoids in zinc deficient subjects induces the MT gene expression which in turn is accompanied by nuclear zinc uptake from the circulation and active MT synthesis. However, the observed low MT concentration in zinc deficient subjects could be the result of the low availability of zinc for the synthesis of the active protein (MT). Although this subject has not been specifically addressed in this dissertation, it is possible to speculate that at early stages of zinc deficiency the tissue MT concentration is increased, but as zinc deficiency persists, a reduction in MT levels as well as circulating zinc levels would evolve.

A study by Nagel and Vallee (1995) proposed an additional function for MT: cell cycle regulation in actively proliferating cells (human colonic cancer cells). They observed the oscillation of MT during the mitotic cell cycle of HT-29 cells with its maximum near the G1/S transition of the cells cycle, at the onset of DNA synthesis. They also claimed that the elevated levels of MT in actively proliferating cells can serve as a marker for proliferation.

Studies on the regulation of MT indicate that much of the regulation occurs at the level of transcription by heavy metals including zinc (Thiele, 1992). The promoter structure of mouse MT-I (one of the MT isoforms) has been most thoroughly investigated and is known to contain multiple metal responsive elements (MRE) responsible for the transcriptional sensitivity of the gene to divalent metal ions (Thiele, 1992). The underlying theory for the role of MRE in MT gene transcription indicates that an interaction occurs

between the inducing metal (eg., zinc) and a specific nuclear factor that through altered conformation recognizes the MRE sequence and participates in the initiation of transcription. Alternatively this factor could bind zinc in the cytoplasm and then enter the nucleus for DNA bindings (Cousins and Lee-Ambrose, 1992). Thus dietary zinc could control MT genes by influencing the intracellular zinc concentration and interaction with metal responsive transcription factors which in turn bind to MRE and initiate transcription.

Role of Zinc in DNA and RNA Synthesis: The impaired rate of growth that accompanies zinc deficiency in mammals could also be a consequence of impairment in the synthesis of DNA, RNA and proteins. Studies were conducted in which whole animals or isolated cells were used to qualify DNA, RNA and/or to measure the incorporation of the radioactive nucleotide precursors (thymidine and uridine) into DNA or RNA under conditions of zinc deficiency (Auld *et al.*, 1975; Duncan and Dreosti, 1976; Dreosti *et al.*, 1981; Duncan and Dreosti,1976). In these studies a consistent observation was that both total DNA and incorporation rates were reduced significantly as a consequence of the zinc deficiency. However, RNA synthesis showed less effects. Many nucleic acid polymerases, as we know, are zinc metalloenzymes. Thus the reduction of uridine incorporation into RNA and thymidine incorporation into DNA of zinc deprived animals or cell cultures could explain the reduced activities of RNA and DNA polymerases.

RNA polymerase contains more than one mole of zinc/mole protein. Hence, it is likely that zinc has both structural and catalytic roles in this enzyme. In an early study by Falchuk *et al.*, (1978), they reported abnormal synthesis of RNA polymerase and altered base composition of the synthesized RNA in a zinc deficient *Euglena gracilis* model. Impaired

mRNA synthesis would lead to altered protein synthesis. In fact, Hicks and Wallwork (1987) have shown a significant depression of protein synthesis in rat liver and rat hepatocytes from zinc deficient rats. They concluded that the defect probably occurred in the tRNA synthetase function suggesting a zinc-dependent activity for this enzyme.

Recent studies on growth requirements for 3T3 cells clearly demonstrated the role of Zn^{2+} for initiation of DNA synthesis. In 1990 Chesters *et al.*, showed impaired thymidine incorporation associated with decreased thymidine kinase activity and a comparable decrease in mRNA by inadequate supply of Zn^{2+}. One year later, in another study by Chesters and Boyne (1991), they further demonstrated the requirement of adequate zinc supply for transition of 3T3 cells from quiescence to S phase. This implies the requirement of zinc for mRNA and protein synthesis involved in the progression of 3T3 cells into S phase. The combination of low Zn^{2+} availability with inhibition of mRNA synthesis or of protein synthesis almost completely returned the cells to a quiescent state. Thus it seems that the lack of zinc primarily restricted gene expression rather than enzyme activation. In fact, Chesters *et al.*, (1995) in their recent investigation on this subject, showed that the inhibition of thymidine kinase activity due to the lack of zinc was associated with increased binding of a specific protein to the gene's promoter in the region between -55 and -83 bp 5' to the transcription initiation site, and inhibition of transcription. This protein whose nature is not known yet, apparently competes with the transcription factors responsible for thymidine kinase expression for the same binding site and by displacing them inhibits the transcription of the gene. Thus in this case the impact of the lack of zinc on thymidine kinase occurred at a pretranslational step. These observations strongly suggest the impairment of DNA, RNA, and protein

ynthesis in zinc deficiency which may subsequently result in some of the observed physiological and immunological alterations presented in this dissertation.

Role of Zinc in Gene Expression: Structural and functional roles of the intrinsic zinc in many DNA and RNA polymerases have been investigated. In this regard, direct zinc removal, and replacement or substitution with other divalent metals have revealed multiple roles for zinc in gene transcription (Chatterji and Wu, 1982).

In an early study by Falchuck *et al.*, (1975), they demonstrated the specific zinc requirement for transition of *Euglena gracilis* from G1 to S, S to G2 and G2 to M phases of the cell cycle where the gradual depletion of the zinc content of the medium lead to the growth arrest. Similarly, a recent study correlated low thymidine kinase mRNA levels in zinc-depleted cells to the presence of two zinc dependent steps during G_1 to S phase transition (Chesters *et al.*, 1993). The role of zinc in gene expression has been also identified at the level of chromatin structure. An early study by Subiranal (1973) demonstrated that the presence of zinc ions facilitated the first phase of chromatin denaturation consisted of the loosely condensed chromatin in the inter-nucleosomal regions. This process is closely related to gene unmasking which is required for synthesis of the enzymes for DNA synthesis. The major change in genetic expression and chromatin structure within the cell cycle occurred in G2 phase immediately before mitosis. Thus the role of zinc in alteration of chromatin structure could explain the arrest of *E. gracilis* cells in G2 as their zinc supply diminished.

In a most recent study conducted by Kimball and his coworkers (1995), the effects of zinc deficiency on hepatic protein synthesis and gene expression of retinol-binding protein and transthyretin (plasma thyroxine-binding protein) in weanling rats was investigated. Their

results showed the inhibition of protein synthesis in livers from zinc deficient rats. This inhibition was accompanied by altered expression of mRNAs in the liver, suggesting that zinc has a vital role in regulating gene expression and protein synthesis.

The role of zinc in gene expression could also explain one of the characteristic signs of zinc deficiency, namely, parakeratosis. This condition, which is the result of poor cell differentiation, could be due to insufficient zinc levels for DNA synthesis and expression of molecules required for normal differentiation of these cells. Several possible mechanisms could underlie the impaired gene expression in zinc deprived animals. First of all, many enzymes involved in nucleic acid synthesis are zinc dependent enzymes, such as DNA polymerase and RNA polymerase. These enzymes have shown to have lower activities in tissues from zinc-deficient animals than from their controls. Second, zinc has been implicated in the stabilization of nucleic acid conformation. Therefore, alteration in tissue zinc concentration could modify the DNA template used for transcription or translation. Finally, zinc-dependent changes in the concentration of other ions, known to have interactions with zinc, such as iron and copper might also alter nucleic acid structure or activity of enzymes involved in their metabolism. For instance, in an early study of Falchuk *et al.*, (1977) on zinc-deficient *E. gracilis*, they observed the accumulation of iron, manganese and copper, and subsequent effect on template specificity and products generated by RNA polymerase specifically due to manganese accumulation.

Another contribution of zinc to gene expression is the zinc requirement of many transcriptional factors for their structural stabilities and their functional activities (Klug and Schwabe, 1995). Many transcription factors include small projections called "zinc fingers"

that are needed for DNA recognition. These structural domains connect transcription factors to their target gene mainly by binding to specific sequences of DNA base pairs (Figure 1). In 1985, Rhodes' lab at the Medical Research Council in Cambridge, England was the first to identify zinc finger structures in a transcription factor of the immature oocytes from the frog *Xenopus laevis*, namely TFIIIA, or factor A (Klug and Rhodes, 1987). Since then more than 200 proteins, many of them transcription factors, have been shown to incorporate such zinc fingers.

The amino acid sequence of TFIIIA was found to be rather unusual, because it contains nine repeat units of about 30 amino acids with conserved cysteine, histidine, and hydrophobic residues (eg., Tyr,Phe,Leu). These sequences are arranged as: Y-X-Cys-X_{2-5}-Cys-X_3-Y-X_5-Y-X_2-His-X_{2-5}-His, where Y represents a hydrophobic residue, X any amino acids, Cys cysteine, and His histidine (Miller *et al.*, 1985; Klug and Schwabe, 1995). The cysteine and histidine side chains coordinate the zinc and the other hydrophobic residues pack to form a hydrophobic core to somehow help stabilize the arrangement (Berg and Shi, 1996), thus making a Cys_2 His_2 type zinc finger domain. The two tightly bound zinc ions in TFIIIA are involved in the multiple roles in the regulation of ribosomal 5SRNA synthesis (Klug and Rhodes, 1987). The structure of each of these zinc finger domains consists of two antiparallel β strands followed by an α helix which interact with the major groove of DNA (Berg and Shi, 1996). A schematic view of a Cys_2 His_2 zinc finger domain is presented in Figure 2.

Over the past decade more than 10 classes of such zinc based domains have been identified and biochemically characterized (Schwabe and Klug, 1994). It has been estimated that between 300 and 700 human genes (about 1% of human genome) encode zinc finger-

containing proteins (Hoovers *et al.*, 1992). A recent study by Bianchi *et al.*, (1992) demonstrated the existence of a $Cys_2 His_2$ zinc finger motif in P2 protamines, one of the sperm nuclear proteins. Zinc is abundant in human sperm nuclei and is presumed to stabilize sperm chromatin through a reversible binding to the thiol groups of mature spermatozoa. Thus the presence of zinc fingers in P2 protamines could contribute to spermatogenesis and fertility, since zinc deficiency leads to infertility.

More relevant to the studies presented in this dissertation is the presence of zinc finger structures in the steroid-thyroid hormone receptor superfamily (Evans, 1988; Carson-Jurica *et al.*, 1990). Cloning and sequencing of the glucocorticoid receptor (GR) has revealed a cysteine-rich region in the DNA binding domain. This region was found to contain two zinc finger structures (Archer *et al.*, 1990; Vallee *et al.*, 1991). While the zinc fingers reviewed so far utilize two cysteines and two histidines to bind zinc in a tetrahedral structure, steroid receptors including GR utilize four cysteines to coordinate zinc (Freedman, 1992; Dahlman-Wright *et al.*, 1992). The requirement of zinc occupancy in the zinc fingers for the DNA binding is absolute, since chelation of incorporated zinc in GR, reduced receptor affinity for DNA binding by over 20 fold (Freeman *et al.*, 1988).

Collectively, the unique characteristics of zinc including its small size, lack of redox activity and its relatively rapid ligand exchange reactions appear to be at least partially responsible for its role as a structural element in nuclear acid-binding or other gene regulatory proteins. Thus, the physiological and immunological alterations observed in zinc deficiency could be partly due to the impaired zinc-dependent molecular activities which are the keys to the biological functions. Alternatively, the reduced zinc availability could exert its effects

indirectly via induction of other molecules (eg., GCs) that are known to be immunosuppressive. The latter will be addressed in Chapter Four of this dissertation.

Figure 1: A schematic view of DNA binding of a transcription factor via zinc finger domains. Three zinc finger structures have facilitated the binding of the transcription factor to the major groove of a DNA molecule (Rhodes and Klug, 1993).

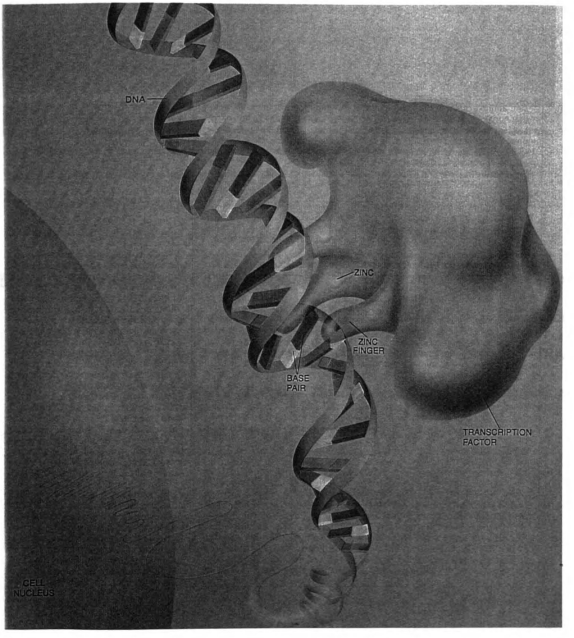
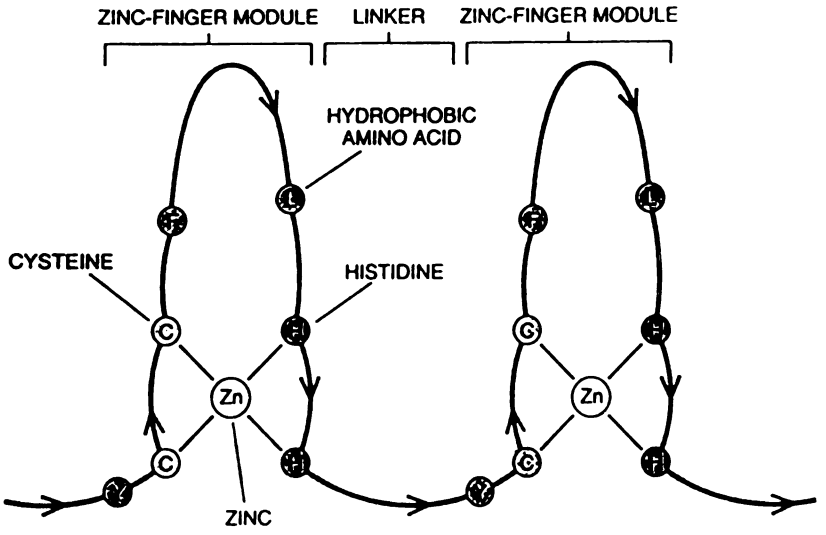
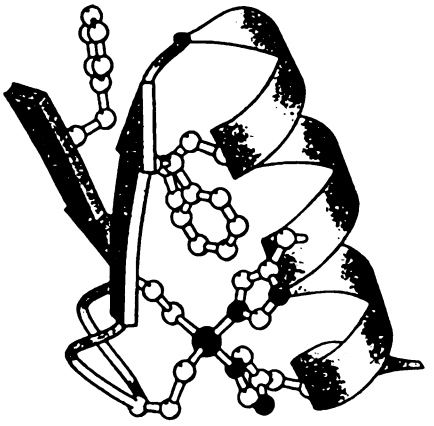

DNA
ZINC
ZINC FINGER
BASE PAIR
TRANSCRIPTION FACTOR
CELL NUCLEUS

Figure 2: Schematic view of the classical zinc finger domain of TFIIIA. **(Top)** Tetrahedral binding of zinc to cysteine (C) and histidine (H) residues along with other amino acid sequences involved in zinc finger domain formation in TFIIIA (Rhodes and Klug, 1993). **(Bottom)** Three dimensional view of the structure of a Cys_2 His_2 zinc finger domain (Berg and Shi, 1996).




```
1                 G I C S F A D C G A A Y N K N W K L Q · A E L C · K E
2 T G E K · P F P C K E E G C E K G F T S L H H L T · R F S L · T E
3 T G E K · N F T C D S D G C D L R F T T K A N M K · K F F N R F F
4 N I K I C V Y V C H F E N C G K A F K K H N Q L K · V F Q F · S F
5 T Q Q L · P Y E C P H E G C D K R F S L P S R L K · R F E K · V E
6 A G · · · · S P C K K D D S C S F V G K T W T L Y L K F V A E C E
7 Q D · · · L A V C · · D V C N R K F R H K D Y L R · D F Q K · T F
8 E K E R T V Y L C P R D G C D R S Y T T A F N L R · S F I Q S F E
9 E E Q R · P F V C E H A G C G K C F A M K K S L E · R F S V · V F
```



LITERATURE REVIEW

SECTION II: Zinc Deficiency and its Effects on Immune System

Zinc Deficiency: As mentioned earlier, zinc is an essential trace element for normal growth and development in both humans and animals (Endre *et al.*, 1990; Walsh *et al.*, 1994; Cousins, 1996). In this regard, the body has developed a very sophisticated control mechanism for the correct delivery and utilization of this trace element to maintain the biological activities efficiently. Therefore, inadequate dietary zinc intake as well as any defect or abnormalities in the utilization, absorption, transportation, and delivery of this molecule to the appropriate tissues could impair homeostasis. One of the most important consequences of this imbalance is the state of zinc deficiency (ZD). It is well established that ZD is a world wide nutritional deficiency which affects both men and women of all ages and socioeconomic status (Prasad, 1991; Walsh *et al.*, 1994). However, the deficiency is prevalent among malnourished patients and children, pregnant teenagers, and elderly with low socioeconomic status (Prasad, 1988). This deficiency predisposes patients to increased risk of infection, and threatens their recovery or even their survival (Prasad, 1988), indicating impaired immune function.

The importance of zinc in human health and the occurrence of dietary ZD in man was first recognized in 1963 when Prasad *et al.*, described a syndrome of dwarfism and hypogonadism in cases from Iran and Egypt (Prasad *et al.*, 1961; 1963). The syndrome described in male Iranian subjects consisted of severe growth retardation, severe anemia, hypogonadism, hepatosplenomegaly, and rough, dry skin (Prasad *et al.*, 1961). Similar clinical manifestations were noted in subjects from the Cairo area in Egypt (Prasad *et al.*, 1963). The only difference between these two groups was that Iranian patients had geophagia whereas almost all the Egyptian cases exhibited *Schistosomiasis* or hookorm infections. It

was hypothesized that lack of zinc was primarily responsible for the observed manifestations in these patients. This was supported by the low concentrations of zinc in plasma, red blood cells (RBC) and hair of these individuals. Furthermore, successful treatment of this syndrome with zinc supplementation confirmed the presence of ZD. The primary cause of ZD in these subjects was related to their poor diet. The main food consumed by these patients consisted of bread and beans which are high in phytate, therefore making insoluble complexes with zinc and inhibiting zinc absorption (Prasad *et al.*, 1961; 1963). In addition to the poor diet, excessive blood loss due to infections and loss of zinc by sweating in hot climates, were several other causes of ZD in these human subjects.

Zinc deficiency is characterized by anorexia and growth retardation as the primary signs in both humans and animals (Chesters and Quarterman, 1970; O'Dell and Reeves, 1989). In humans the manifestations of ZD include poor appetite, growth retardation, male hypogonadism, mental disturbances, delayed wound healing, and skin disorders. If ZD persists, the symptoms become more pronounced with the addition of recurrent infections due to the depressed immune system. If untreated, the ZD becomes fatal (Prasad, 1988; Mills, 1989; Aggett, 1989; Hambidge, 1989). In experimental animals the manifestations include anorexia, growth cessation, gastrointestinal malfunction, diarrhea, dermatitis (eg., parakeratosis) and impaired immunocompetence (Carlomagno and McMurray, 1983; Hambidge *et al.*, 1986).

The importance of zinc in numerous biological functions and the deleterious effects of its deficiency have initiated a growing body of literature on the etiologies of this nutritional deficiency which are summarized below.

Etiology of Zinc Deficiency: Since diet is the major source of zinc intake, a common cause of mammalian ZD is underlined by improper diet and insufficient food intake (O'Dell and Reeves, 1989; Walsh *et al.*, 1994). In spite of the ubiquity of zinc in almost every tissue, there is no known body store for zinc, as there is for iron; therefore zinc must be supplied in the diet continuously. The recommended daily allowance (RDA) of zinc in human is about 10-15 mg (Walsh *et al.*, 1994).

In addition to adequate dietary zinc intake, dietary composition contributes to the bioavailability of zinc for intestinal absorption. For instance, severe ZD is prevalent in populations whose diet is mainly consisted of large quantities of plant proteins (eg., unleavened grain products) containing phytate which is known to form insoluble complexes with zinc and decrease the absorption of zinc (Solomons and Cousins, 1984; Sandström and Lönnredal, 1989). As described previously, early studies in southern parts of Iran and in Egypt documented the excessive intake of plant proteins mainly in form of unleavened bread as the principal cause of ZD in these areas (Prasad, 1991; 1995). Furthermore a recent study on peri-urban Guatemalan school children with low socioeconomic status and poor diet revealed a state of ZD in these subjects (Cavan *et al.*, 1993). The main food consumed by these children were corn tortillas and black beans which contain high amounts of phytate, dietary fiber and calcium, all of which are known to inhibit zinc absorption. Selected zinc indices such as hair, plasma zinc concentrations and the activity of alkaline phosphatase in plasma and red blood cell membranes were examined and were shown to be diminished in both sexes, being more pronounced in boys. Upon zinc supplementation, these conditions were corrected to the normal levels.

Acrodermatitis enteropathica (AE), a genetic disorder in zinc assimilation, also results in ZD. AE is a rare childhood disease of high morbidity and mortality and is inherited as an autosomal recessive trait, affecting the sexes equally (Dillaha *et al.*, 1953; Moynahan, 1974). The role of zinc in this genetic disorder was recognized when Moynahan and Barnes (1973) used zinc supplementation on a case with this disorder. The patient showed a remarkable recovery after administration of zinc. This observation was rapidly confirmed by Moynahan in 1974. The possible mechanism responsible for ZD in AE has been related to impaired intestinal uptake (77% decrease) and transfer of zinc (Aggett *et al.*, 1981; Weismann *et al.*, 1979; Atherton *et al.*, 1979). In a most recent study by Grider and Young (1996), it was noted that the impairment in zinc uptake in AE patients is not restricted to intestinal mucosal cells. Using fibroblasts from AE patients, they demonstrated significant decrease in zinc content, 5'nucleotidase activity (a zinc metalloenzyme), and altered zinc uptake kinetics compared with normal fibroblasts. These data suggested that perhaps the AE mutation affects the number of functional zinc transport proteins by either reducing their total number or affecting their function. This condition is associated with low plasma zinc, growth retardation, hypogonadism, skin lesions, bowl disorders, central nervous system malfunction and frequent immunodeficiency (Moynahan, 1974; Van Wouwe, 1989). The close resemblance between AE manifestations in human subjects and symptoms of ZD in animals (eg., adult mice) support the use of animal models for the study of ZD in humans.

ZD has also been reported in patients with sickle cell anemia, an inborn error of hemoglobin metabolism which results in red cell sickling and continuous hemolysis, with an accompanying high rate of infections (Ballester and Prasad, 1983; Prasad, 1993). An early

study by Prasad and colleagues (1978) reported decreased zinc in plasma erythrocytes and hair of these patients with concomitant increased urinary zinc excretion. The continued hyperzincuria and hemolysis in these patients and the existence of zinc as an important constituent of erythrocytes may have been responsible for the state of ZD.

In addition to dietary ZD and inherited disorders in zinc absorption, there are several disease states which may lead to ZD. The diseases and the possible explanations for the presence of ZD (indicated in parenthesis) in each case are summarized as: chronic renal disease (proteinuria and failure of tubular reabsorption); chronic alcoholism (increase in renal clearance of zinc and hyperzincuria); gastrointestinal disorders (insoluble zinc-protein and zinc-fat complex formation); cirrhosis of the liver (abnormal zinc assimilation and hyperzincuria); diabetes mellitus (increased urinary losses of zinc); various neoplastic diseases (anorexia and starvation); parasitic infections (blood loss); burns (losses in exudates); psoriasis (loss of skin epithelial cells and the massive formation of new cells); and AIDS (reduced appetite, decreased dietary intake, gastrointestinal malabsorption, and frequent acute and chronic infections) (Prasad, 1979; Pai and Prasad, 1988; Endre *et al.*, 1990; Shippee *et al.*, 1992; Odeh, 1992; Walsh *et al.*, 1994).

Collectively, this information supports the critical role of zinc in many biological functions, with zinc deficiency resulting in numerous physiological complications including impairment of immune function. The studies provided herein have utilized the induction of dietary ZD in mice as a general model to emphasize the importance of zinc in immune integrity and to provide significant information on the status of various immune components in ZD.

Zinc Deficiency and the Immune System: The interrelationship between nutritional deficiencies and the immune response has been well established in both human and animal studies (Good and Lorenz, 1992; Sherman, 1992; Fraker *et al.*, 1993; Prasad, 1995). Being a dynamic system with constant development, differentiation, and proliferation, the immune system requires a continuous flow of nutrients for its proper function. Among nutritional elements zinc has been recognized as an essential trace element for the development and integrity of the immune system, as discussed previously (Endre *et al.*, 1990; Sherman, 1992; Aggett and Comerford, 1995). In fact numerous clinical and experimental observations have documented the deleterious effects of ZD on immune function (Keen and Gershwin, 1990; Prasad, 1991; Dardenne, 1993; Fraker *et al.*, 1993; Walsh *et al.*, 1994).

As mentioned previously, the importance of zinc in immunity was first recognized with the discovery of human ZD due to a poor diet (Prasad *et al.*, 1963). Dwarfism, testicular atrophy, low zinc concentrations in plasma, hair, and erythrocytes, and increased susceptibility to infections were characteristic complications in these patients. The reversibility of these clinical manifestations upon zinc supplementations established the presence of ZD in these individuals.

Further evidence relating zinc deficiency to impaired immunity have come from patients with *Acrodermatitis enteropathica*, a classical model for zinc deficiency. Early studies by Moynahan and Barnes (1973) described a 2-year-old girl with severe AE and several immunological complications such as skin lesions, gastrointestinal disorders, decreased delayed hypersensitivity reactions, and reduced numbers of peripheral T and B cells. This lymphopenia is a condition accompanying ZD. Upon zinc supplementation all the

symptoms were corrected. The immunological abnormalities generally described in patients with AE are thymic atrophy, lymphopenia, reduced proliferative response to mitogens, decreased natural killer (NK) cell activity, decreased neutrophils and monocytes chemotaxis activity, deficient thymic hormone activity and increased susceptibility to infections (Chandra 1980; Van Wouwe, 1989; Endre *et al.*, 1990). The presence of ZD in these patients is thought to be due to congenital malabsorption of zinc.

The development of experimental dietary ZD in humans was an advancement in the characterization of specific physiological and immunological alterations caused by mild ZD (Abbasi *et al.*, 1980; Prasad, 1991; Rabbani *et al.*, 1987). Consumption of a semisynthetic soy-protein based diet (3-5 mgZn/day) by male volunteers for 28 weeks resulted in oligospermia, decreased serum testosterone and thymulin activity, decreased lean-body mass, and decreased interleukin II (IL-2) production. In addition, diminished NK activity, and alterations in T-lymphocytes subpopulations with selective decrease in T-helper cells were noted. Zinc supplementation of 30 mg/day for 20 weeks reversed all the above symptoms (Rabbani *et al.*, 1987).

The incidence of ZD and immunological abnormalities have been also reported in children with severe protein calorie malnutrition (PCM). Indeed lower serum zinc levels are often noted in PCM. The common features observed in human ZD and PCM include: anorexia, diarrhea, growth retardation, thymic atrophy, lymphopenia, reduction in lymphoid tissues, impaired cell-mediated and humoral-mediated immunities, and increased susceptibility to infections. These manifestations as well as reduction in plasma, muscle, and liver zinc levels led to the suggestion of the role of ZD in the development of PCM. As expected, zinc

therapy in children with PCM reversed the manifestations and resulted in thymus growth in these subjects (Golden *et al.*, 1977; Kuvibidila *et al.*, 1993).

Children with Down's syndrome, trisomy of chromosome 21, also demonstrate a marginal zinc deficiency associated with impairment of thyroid functions, reduced thymulin activity, reduced neutrophil chemotaxis function and subsequent increased susceptibility to infections (Licastro *et al.*, 1992). Improved immunological efficiency observed in these subjects after zinc supplementation confirms the role of ZD in the pathogenesis of some of these immune defects (Licastro *et al.*, 1992).

The role of low bioavailability of zinc on the impairment of immune system, has also been reported in patients with rheumatic heart disease (RHD), cystic fibrosis, leukemia, and chronic uremia. The impaired cell-mediated immunity, decreased delayed hypersensitivity responses, decreased thymulin activity, and significant low serum zinc levels are the common immunological defects in these patients (Gorodestky *et al.*, 1985; Consolini *et al.*, 1986; Gupta *et al.*, 1992; Bonomini *et al.*, 1993; Mocchegiani *et al.*, 1994; Mocchegiani *et al.*, 1995).

The role of zinc in cellular immunity, or the thymus-dependent immune functions, has been recently related, in part, to the regulatory role of thymulin in T-cell differentiation and maturation (Prasad *et al.*, 1988). Thymulin (previously known as facteur timique serique, or FTS), one of the best known thymic hormones, is a zinc dependent hormone synthesized by the epithelial cells of the thymus and is adversely affected by ZD. Zinc is required to confer biological activity to thymulin, which has a modulating action on cell-mediated immunity. The zinc-unbound form is inactive and can inhibit the active form possibly via competing for

the thymulin receptor (Fabris *et al.*, 1984; Prasad *et al.*, 1988). It has been shown that with ZD the thymic peptide, thymulin, is synthesized and secreted in normal amounts, but only a fraction of it binds zinc ions and becomes active. However, *in vitro* addition of zinc ions causes an increase in the active form of thymulin (zinc-bound thymulin) which in turn enhances thymocyte responses to mitogens (eg., phytohemagglutinin, PHA; concanavalin A, ConA) (Saha *et al.*, 1995).

In addition to disease states, many physiological conditions in which the state of zinc deficiency is observed are accompanied by altered immunological functions. For instance, during aging there is a considerable loss in both zinc status, possibly due to reduced intestinal zinc absorption, and immunological function particularly cell-mediated immunity (Chandra, 1990; Ripa and Ripa, 1995). Several investigators have shown improvement of immune function-specially delayed type hypersensitivity reactions (DTH) and significant restoration of serum thymic hormones-activities in elderly subjects after low dose zinc supplementation (20-30 mgZn/day) (Bunker *et al.*, 1987; Prasad, *et al.*, 1993; Boukaiba *et al.*, 1993; Bogden *et al.*, 1994; Bogden, 1995). This indicates that zinc supplementation in elderly populations provides significant immunological benefits. Furthermore, it should be noted that the zinc requirement is higher during periods of rapid growth such as infancy, childhood, adolescence, and pregnancy, thereby putting these populations at a greater risk of ZD (Moore *et al.*, 1984; Hambidge *et al.*, 1986; Prasad 1995; Ripa and Ripa, 1995).

Parallel to human studies, dietary studies in animals have also shown similar patterns of impaired cellular and humoral-mediated immunity as well as thymic atrophy and lymphopenia due to zinc deprivation and the restoration of immune function by zinc repletion.

Using a murine model in a 30-day dietary zinc studies, Fraker and colleagues initiated a series of investigations on the effects of suboptimal dietary intake on immunity. The following information is a review of these accomplishments as well as some studies by other investigators which in parallel explain the immune capacity of the murine system in zinc deficiency.

In 1977, Fraker *et al.* demonstrated that 4-week dietary ZD (0.5 μgZn/g diet) caused rapid thymic involution (>60%) in young adult (6-week old) A/J mice. In 6 weeks, mice fed ZD diet became athymic. When the antibody-mediated response of these mice to sheep red blood cells (SRBC) was evaluated, ZD mice produced only 10% of the number of anti-SRBC antibody producing cells (PFC; plaque forming cells) generated by zinc adequate (ZA) control mice. Reconstitution of the ZD mice with thymocytes from normal mice restored their response and generated 61% as many plaques as the control mice. These data clearly indicated that ZD greatly affected T-cell helper function. In a similar dietary zinc study, Fernandes and coworkers (1979) investigated the immune response of different strains of young adult mice (A/J, C57BL/Ks, and CBA/H) to deficiencies in zinc. When placed on ZD diet, these mice showed loss of body weight, significant involution of thymus, and low serum zinc levels within 4-8 weeks after initiation of diet. Almost 50% of the mice on ZD diet developed severe skin lesions on tail and paws and diarrhea. Furthermore, defective development of both direct and indirect PFC after *in vivo* immunization with SRBC, depressed T-killer cell activity against *in vivo* immunization with tumor cells, and low NK cell activity were observed in ZD group. These data confirmed the previous findings on the

impairment of T-cell dependent immune functions in ZD and demonstrated similar immunological response from different strains of mice to this nutritional deficiency.

To further characterize the effects of ZD on antibody-mediated responses of young adult mice, the capacity of ZD mice to respond to T-cell independent (TI) antigens were also evaluated (Jardieu and Fraker, 1990). The TI antigens primarily elicit responses from macrophages and B-cells with less involvement of T-helper cells. Furthermore, response to TI antigens develops at different stages of B-cell development. Trinitrophenyl conjugated lipopolysaccharide (TNP-LPS), a TI-class 1 antigen, stimulates immature B-cells; whereas, TNP-Ficoll, a class 2 TI antigen, stimulates more mature B-cells (Fraker *et al.*, 1984). After a 28-day period of suboptimal intake of zinc, the deficient mice exhibited only 32% as many total splenic anti-TNP PFC to TNP-LPS and 50% as many PFC in response to TNP-Ficoll as compared to ZA fed mice at day four after immunization. By day 5, the deficient mice could only produce 28,000 anti-TNP PFC per spleen compared to 69,000 PFC produced by ZA mice. These data represented a significant reduction in host defense capacity. The kinetics of the response of the ZD mice to each antigen was delayed by two days from the optimal time of response to the antigen (day 3). In evaluation of the proportion of splenocytes responding to antigenic challenge (PFC/10^6 viable splenocytes) over time (3, 4, 5 days post immunization), the optimal response of the deficient mice was again delayed by two days compared to the response of ZA group. However by day 5, the number of PFC/10^6 splenocytes had increased in the ZD group, being equivalent to ZA group at their optimum day (day 3). This suggested that the residual B-cells of ZD mice were functional, with perhaps slower rate of activation and proliferation in response to antigenic stimuli. In a

similar dietary zinc study conducted by Moulder and Steward (1989), mice fed ZD diets had reduced numbers of T-cells and T-cell subsets, decreased antibody and cell-mediated responses to T-cell dependent (TD) and TI antigens as well as decreased IL-2 production. Overall, the significant reduction in antibody-mediated responses to either TD or TI antigens indicate a substantial loss in host defense capacity in zinc deprived mice.

As indicated earlier susceptibility to infection is also pronounced in ZD. In this regard, Fraker *et al.*, (1982) investigated the effects of this deficiency on host resistance to infection by *Trypanosoma cruzi*, an intracellular parasite causing Chagas' disease in South America. The extreme vulnerability of ZD mice to this pathogen was demonstrated by a substantial increase in blood parasites in ZD mice (50 fold) compared to controls (infected mice in restricted or zinc adequate fed groups). Furthermore, by 22 days post-infection, almost 80% of the infected ZD mice died, whereas there was no mortality among either the uninfected ZD or the infected ZA mice. These data clearly demonstrated the synergistic interaction between ZD and *T. cruzi* on the observed death rate.

Since mononuclear phagocytic cells (MNPs) are the first line of defence against *T. cruzi*, the follow-up experiments were designed to evaluate MNP function. In this case, it was shown that the inability of the ZD mice to defend against infection by *T. cruzi* was most likely due to the defective phagocytic and microbicidal activities of resident macrophages (Wirth *et al.*, 1989). At time zero after infection, the percentage of macrophages engaged with *T. cruzi* and the number of *T. cruzi* per 100 macrophages was substantially reduced in the ZD group. Twenty four hours after infection, macrophages from the ZD group were unable to destroy as many parasites as macrophages from the ZA group. In other words,

there was a reduction (20-50%) in the number of parasites per 100 macrophages in the ZA group but not in the ZD group. Interestingly, pre-incubation of MNPs from the ZD mice for one hour with zinc chloride (10 μg/ml) restored all the functions (Wirth *et al.*, 1989). Thus it was evident that the macrophages from ZD mice had some functional processes that were impaired due to the low availability of zinc. This defective function was later related to the oxygen burst activity which is thought to be heavily dependent on metals such as zinc (Cook-Mills and Fraker, 1993b).

The profound effects of zinc deficiency on immune integrity against pathogens has been further emphasized in recent murine studies in which animals fed different dietary zinc levels were challenged with a variety of intestinal parasitic worms (Fenwick *et al.*, 1990a; 1990b; Nawar *et al.*, 1992). In all cases zinc deficiency depressed both humoral and T-cell mediated immunity, enhanced establishment of the parasites and impaired expulsion of the parasite from the intestine. Zinc supplementation, however, restored rapidly the ability to expel the infection by few days postinfection (Fenwick *et al.*, 1990a; 1990b).

In contrast to these studies, when Minkus and coworkers (1992) challenged the mice with *Heligmosomoides polygyrus*, (an intestinal nematode), their results showed no significant differences between zinc restricted fed (5 mg/kg) mice and control mice with respect to cell mediated immune response, worm proliferation (worm numbers), and egg production. However, plasma zinc concentrations were significantly lower in the zinc restricted fed mice. These outcomes were reversed when the same examinations were evaluated in mice made severely zinc deficienct (<0.75 mg/kg in the diet) (Shi *et al.*, 1995). These findings clearly indicate that marginal intake of dietary zinc (5 mg/kg) is not sufficient to affect the survival

of the intestinal nematodes. Furthermore, the low plasma zinc cannot be used by itself as an index for zinc deficiency, since many physiological alterations (including infections) causes plasma zinc depletion (Walsh *et al.*, 1994).

As with ZD human subjects, delayed type hypersensitivity reactions (DTH) in which macrophages and T-helper cells are primarily involved are also affected in ZD mice. When DTH response was measured in mice, ZD animals gave a very poor response (50% less than ZA controls). However, after 21 days of nutritional repletion, re-feeding the ZD group with ZA diet (55 μgZn/g diet) resulted in normal DTH response in previously ZD mice (Fraker *et al.*, 1982).

With regard to these observations, it appeared that in spite of multiple defects in immune system due to ZD, nutritionally deficient animals had the capacity to repair their immune system when re-fed nutritionally adequate diets. To confirm this, 5-week old A/J mice were fed ZD diet (<1 μgZn/g diet) for 31 days (Fraker *et al.*, 1978). Thymuses from ZD mice were one-third of normal size with preferential involution of the cortex. The direct PFC (IgM response) and the indirect PFC (IgG response) produced per mouse spleen to SRBC immunization were 34% and 18% of the normal, respectively. The deficient mice were then re-fed ZA diet (50 μgZn/g diet) and the degree of restoration of the thymus as well as regeneration of T-cell helper function were evaluated at 1, 2, and 4 weeks. By 4 weeks, the thymus weights and antibody-mediated responses to SRBC were normal. Thus it was obvious that ZD young adult mice had the capacity to restore T-cell dependent antibody-mediated responses after dietary zinc repletion.

In a subsequent study in which the functional capacity of residual lymphocytes from ZD mice was investigated, cultured splenic T-cells in autologous sera from ZD mice gave normal proliferation and adequate IL-2 activity when stimulated with Con A. Furthermore, splenic B-cells from ZD mice which were stimulated *in vivo* with SRBC produced significantly lower number of PFC (50%-70% depression based on the degree of deficiency) compared to the ZA group. However, the proportion of B-cells responding as determined by the number of PFC/10^6 viable splenocytes remained unchanged in ZD mice. It was also demonstrated that similar amounts of IgM and IgG antibodies per activated B-cell were produced by all cells from all dietary groups. In addition, the average number of splenocytes in MZD and SZD was reduced by 43% to 47%, respectively, compared to ZA control. These findings emphasized that many of residual lymphocytes of the deficient mouse were functional and correlated the impaired immunity in ZD to lymphocytopenia (Cook-Mills and Fraker, 1993a).

The presense of lymphopenia and defective host defense in ZD prompted the investigations on the degree of sensitivity of lymphoid subsets to the effects of this deficiency. In this regard, the spleen, which contains predominantly mature lymphocytes of different subsets, was used to evaluate the proportion or the phynotypic distribution of T and B cells (King *et al.*, 1991). In spite of significant reduction in the total numbers of splenic lymphocytes (~50% loss), it seemed that ZD had no significant effect in the distribution of the more mature lymphoid subsets residing in the spleen. Marginally ZD mice showed a normal ratio of T cells to B cells, and severely ZD mice demonstrated a 20% increase in the overall ratio of T helper to T suppressor cells. These data, as well as other human and animal

studies in which thymic atrophy and lymphopenia were predominantly observed in ZD subjects (Moulder and Steward, 1989; Fraker *et al.*, 1993; Kuvibidila *et al.*, 1993), suggested that reduction in absolute number of lymphocytes involved in immune response was the principal cause of reduced host defense. Knowing that zinc plays a significant role in the functional activities of many enzymes involved in cell proliferation and growth, alteration of lymphopoiesis in ZD was possible. Thus, extensive studies on the BM, as the site of lymphopoiesis in adult mammals, were initiated. In fact, Chapter Two represents the first detailed study on the status of developing B-lymphocytes in the marrow of ZD mice. This was a collaborative work with Dr. Louis King, a senior investigator in our laboratory. As will be shown, this study demonstrates a significant loss (40-90%) in the proportion of nucleated marrow B-cells with substantial sensitivity of more immature B-cells (35%-80%) depending on the severity of the deficiency in ZD animals. These observations mediated more close examination of B-lymphopoiesis in earlier stages of development (eg., progenitor and precursor B-cells) to further specify the pattern of alterations in these populations. This will be addressed in Chapter Three.

Thus, the overall pattern of immunological changes in ZD animals shows a high correlation with alterations observed in zinc deprived human subjects. Furthermore, both animal and human studies once again emphasize the multiple roles of zinc in the developing immune response. The summary of above findings mainly indicate that a substantial portion of the impairment of immune response was due to the lymphopenia and decreased number of lymphocytes engaged in defense rather than functional disruption. This reduction in lymphocytes numbers could be either due to susceptibility of these cells to the less availability

of zinc, as zinc is critical to cellular and metabolic activities and survival of the cells, or due to alterations in lymphopoietic processes responsible for generation of these cells. In addition to the direct effects of zinc deficiency by itself, the elevation of glucocorticoids in zinc deficiency, which is known to be immunosuppressive, could be an additional significant factor in depressed cellularity of the immune system. These are some important possibilities that unfortunately have been neglected in the study of nutritional-immunology and immunopathology. However, this study has tried to focus and to evaluate the aforementioned possibilities in the suppression of lymphopoiesis in zinc deficiency. Since BM is the primary site of lymphopoiesis in adult mammals, and thymus is the site of T-cell maturation, these tissues were utilized in this work. Chapters Two and Three will exclusively demonstrate the effects of ZD on B-lymphopoiesis in the marrow and the extent of the susceptibility of each subpopulation of B-lymphocytes to the effects of this deficiency. Furthermore, the status of T-cell maturation in the thymus of dietary ZD mice will be addressed in Chapter Five.

LITERATURE REVIEW

SECTION III: Phenotypic Characterization of B and T Lymphocytes at Different stages of development in Murine System

Introduction: During mammalian ontogeny, the lymphohemopoietic lineages are generated sequentially in yolk sac, fetal liver, fetal spleen, and bone marrow (BM) which then becomes the major site of lymphohemopoiesis during postnatal life (Kincade, 1981; Ikuta *et al.*, 1992; Marcos *et al.*, 1994). The process of lymphohemopoiesis is believed to be regulated by the BM microenvironment. The hematopoietic microenvironment in the bone marrow, which consists of adherent stromal cells such as endothelial cells, fibroblasts, and dendritic cells as well as various cytokines and adhesion molecules, supports a continuous proliferation and differentiation of lymphohematopoietic cells of multilineages, in addition to the pluripotent hematopoietic stem cells (PHSCs) (Dorshkind, 1990; Ikuta *et al.*, 1992). These stem cells, with an estimated frequency of 0.01-0.005% of all nucleated cells in the BM (Tsai *et al.*, 1994), have the capacity for extensive self-renewal, and the ability to rescue lethally irradiated animals by giving rise to all different blood cell types which are the cellular components of the immune system (Figure 1) (Heimfeld and Weissman, 1992; Ikuta *et al.*, 1992).

Although, the question of whether T and B lymphocytes arise from a common lymphoid progenitor or directly from multipotent hematopoietic stem cells is still unclear, it is certain that their site of maturation is different. Progenitor T-cells are generated in the BM, and then migrate into the thymus, where they proliferate and differentiate into mature T-cells. Mature T-cells which reside in the thymic medulla leave this tissue through veins and lymphatic vessels and join the circulating lymphocyte pool (von Boehmer, 1992; Kruisbeck, 1993; Pawlowski and Staerz, 1994). In contrast, B progenitors in mammals are generated and remain in the BM, where they differentiate into mature immunoglobulin (Ig) bearing

B-cells via a complex series of steps. These maturation steps involve lymphocyte-stromal cell interaction, Ig gene rearrangement, and surface expression of Ig molecules (Osmond, 1993; Melchers *et al.*, 1995). The mature B-cells then enter the blood stream and migrate into the secondary lymphoid organs (eg., spleen, lymph nodes) where they participate in immune responses. Thus, since all development and maturation of B-lymphocytes occurs in the BM, it is easy to assess the status of different stages of B-cell development and the function of BM lymphopoiesis in ZD in which lymphopenia is predominantly observed. Thus to comprehend the status of lymphopoiesis in ZD, it is necessary to review the stages of lymphocytes development as well as associated cellular and molecular events, all of which will be discussed in the following sections.

B-cell development in the BM:

As mentioned earlier, adult mammalian B-cells, which comprise 23-25% of the heterogenous BM population, are predominantly generated and mature in the primary lymphoid organ, BM (Osmond *et al.*, 1994). Although early stages of B-cell development are not well defined, a combination of cellular and molecular events such as Ig gene rearrangements and gene expression, the expression of specific surface and molecular markers, as well as growth requirements have been utilized to characterize the sequential steps in B-lymphopoiesis in the BM. In this regard a few models have been proposed, some of which will be discussed here. However, with the use of Hardy's scheme of B-cell development (1991) in this dissertation, the main emphasis will be given to this system.

The most primitive cells of the B-lineage are called progenitor B (pro-B) cells. These B lineage restricted cells, which comprise 4-6% of the total nucleated bone marrow cells,

retain Ig genes in the germline configuration and have the capacity to differentiate into mature B cells expressing diverse antigen receptors (Hardy *et al.*, 1991). These progenitor B-cells that are believed to start gene rearrangement by joining the D (diversity) and J (joint) segments of the Ig heavy chain ($D\text{-}J_H$), subsequently become large precursor B-cells (pre-B) in which the variable(V) segment of the Ig heavy chain (IgH) is joined to the previous $D\text{-}J_H$ complex ($V\text{-}D\text{-}J_H$) (Tarlinton, 1994). This recombination generates a complete IgH chain gene (μ), shown in Figure 2, which is expressed in the cytoplasm of pre-B cells (Cμ) and is the characteristic of the pre-B stage of development. This population accounts for 8-12% of the total BM cells. Furthermore, studies on early B-cell development have identified the association of a pseudo light chain, known as surrogate light chain (ψL), with μ heavy chain (μH) of Ig in place of the conventional light chain (κL or λL) of Ig on the surface of pre-B cells (Karasuyama *et al.*, 1994). This protein is composed of two polypeptide chains and encoded by the Vpre-B and λ5 genes (Rolink *et al.*, 1994). the Vpre-B gene has sequence homology to the V region of the IgH and IgL chain genes and the λ5 gene has homology to the J and C regions of Ig λL chain gene (Rolink *et al.*, 1994). The expression of these two genes is largely restricted to developmental stages before light chain gene rearrangement, namely the pro-B cell stages and the large pre-B (Melchers *et al.*, 1993; Li *et al.*, 1993; Karasuyama *et al.*, 1994). *In vitro* studies on the significance of the μH-ψL complex formation have assigned signal transduction activities to this complex, since its expression was accompanied by an increase in intracellular Ca^{++} levels as well as tyrosine phosphorylation of intracellular proteins (Misener *et al.*, 1991). Moreover, using λ5-deficient mice generated by gene targeting technique, it was shown that B-cell development of early stages (pro and pre-B

cells) was disrupted in these mice (Kitamura *et al.*, 1991). In the next step of Ig gene rearrangements, the light chain gene rearrangement ($V\text{-}J_L$) occurs in small pre-B cells carrying cytoplasmic μ heavy chain. The rearrangement of light chain begins with the κ gene segment. If the rearrangement is successive, the light chain rearrangement would stop with κ being the predominant light chain of the Ig molecule. If the rearrangement of both κ alleles is nonproductive, the λ gene segment undergoes rearrangement. Successful light chain rearrangement together with μ heavy chain results in the expression of a complete Ig molecule (IgM) on the surface of immature B-cells. Further differentiation of immature B-cells will lead to the coexpression of IgM and IgD on the surface of mature B-cells (Rolink and Melchers, 1991; 1993). The mature B-cells will then leave the BM and migrate to the spleen and lymph nodes where they could be stimulated by foreign antigens (Picker and Butcher, 1992; Butcher and Picker, 1996). Upon activation of B-cells by antigens, IgD is down regulated and the cells develop into memory cells and antibody secreting plasma cells with IgM secretion as the early primary response (Roes and Rajewsky, 1993; Desiderio, 1994). Recent studies on the structure of the B-cell membrane bound Ig molecules have demonstrated that these Ig receptors are non-covalently associated with a heterodimer of two transmembrane proteins, namely Ig-α and Ig-β (Hombach *et al.*, 1988; 1990; Campbell and Cambier, 1990). In fact, several studies have concluded that the IgM molecules will be expressed on the surface only when associated with Ig-α/Ig-β chains, becoming a complex receptor analogous to T-cell receptor complex (CD3-αβ/TCR) (Sakaguchi *et al.*, 1988; Hombach *et al.*, 1988; Kashiwamura *et al.*, 1990). The Ig-α and Ig-β proteins are the product of mb-1 and B29 genes, respectively (Hombach *et al.*, 1990; Matsuuchi *et al.*, 1992).

These

antibo

Nomu

these

Camb

bearin

as exp

et al

devel

fractio

surfac

IgD, i

order

Fracti

B-cell

imma

Fracti

dissert

3. Wh

growth

depend

These transmembrane glycoproteins seem to be involved in signal transduction since antibodies against mb-1 causes Ca^{++} influx in pre-B lymphoma cells (Yamanishi *et al.*, 1991; Nomura *et al.*, 1991; Matsuo *et al.*, 1993). The heavy chain of IgD is also associated with these heterodimer glycoproteins, however, with slightly different Ig-α size (Campbell and Cambier 1990; Chen *et al.*, 1990).

Besides Ig gene expression, the progression of early immature B-cells to mature Ig bearing B-cells is also marked by acquisition or loss of B-cell differentiation markers as well as expression of specific genes and their products required for B-cell development. Hardy *et al.*, (1991) in their recent investigations on determination of early stages of B-cell development were able to subdivide B-lineage cells of the marrow into several distinct fractions or subpopulations. This investigation was based on flow cytometric analysis of cell surface markers such as CD43 (S7) or leukosialin, BP-1, heat stable antigen (HSA), IgM, IgD, immunoglobulin gene rearrangement and growth requirements. Thus, they proposed an ordered differentiation pattern of B lineage cells as: Pre-pro B ($B220^+CD43^+HSA^-BP\text{-}1^-$; Fraction A), early Pro-B ($B220^+CD43^+HSA^+BP1^-$; Fraction B), late Pro-B plus to precursor B-cells ($B220^+ CD43^+HSA^+BP\text{-}1^+$; Fraction C), small pre-B ($B220^+CD43^-IgM^-$; Fraction D), immature B ($B220^+CD43^-IgM^+IgD^-$; Fraction E), and mature B ($B220^+CD43^-IgM^+IgD^+$; Fraction F) (see Figure 3). The modified version of this scheme was utilized in this dissertation to identify the subpopulations of pro and pre-B cells, as demonstrated in Figure 3. When the growth requirement of each isolated subset was evaluated, they found that the growth and proliferation of cells from the earliest fraction (Pre-Pro B) was absolutely dependent on contact with a stromal layer whereas later fractions (early Pro-B, late Pro-B,

large Pre-B) could proliferate in the presence of soluble mediator interleukine-7 (IL-7) alone. This data is in agreement with earlier studies from Dorshkind Laboratory (1985; 1990) which showed the absolute dependence of earliest B lineage progenitors on direct connection with stromal layer and the proliferation of large pre-B cells on rIL-7 alone (without the presence of stromal cells). Furthermore, using a deletional PCR assay, which permits the amplification of DNA sequences that are normally deleted upon gene rearrangement, they noticed that cells of the earliest fraction (Pre-Pro B) showed no Ig rearrangement, and as cells progressed toward intermediate (early Pro-B) and late fractions (late Pro-B, large Pre-B) they possessed D-J_H rearrangements (Hardy *et al.*, 1991).

Several years later (1993) Li and his colleagues from Hardy's laboratory confirmed and extended the aforementioned observations by identifying the stage-specific expression of genes involved in Ig gene rearrangements such as terminal deoxynucleotidyl transferase (TdT), recombination activating genes (RAG1, RAG2) (Ferguson *et al.*, 1994) and genes that associate with heavy chain, eg., λ5, and Vpre-B (Melchers *et al.*, 1993). In this complementary study they detected high expression of TdT in early Pro-B, late Pro-B, and large Pro-B, and its absence in small pre-B cells. TdT, an intranuclear zinc metalloenzyme, plays a role in generating immunological diversity by addition of non-germline encoded nucleotides (N regions) at the V-D and D-J junctions of IgH chain genes (Landau *et al.*, 1987). Presence of such N regions in IgH chain gene and its absence in light chain gene (V-J_l) correlates with the detection of TdT early in B cell differentiation and its absence in later stages of development reported by Li *et al.*, (1993) findings. The other two genes, λ5 and Vpre-B, which form the surrogate light chain (Karasuyama, *et al.*, 1994), showed the same

expression pattern, being detected in early Pro-B, late Pro-B, and large Pre-B cells and absent when light chain gene rearrangement occurs. In contrast with these three genes, the co-expression of RAG-1 and RAG-2 enzymes responsible for heavy and light chains gene rearrangements (Ferguson *et al.*, 1994) was significant in small pre-B , where much of the light chain gene rearrangement takes place. Moreover, these enzymes were also detected in early and late pro-B cells (Li *et al.*, 1993).

Parallel to Hardy's scheme of B-cell development, Osmond and his colleagues in a series of studies have defined stages of B-cell differentiation using a combination of B-lineage associated surface and molecular markers as well as mitotic arrest techniques (Park and Osmond 1987; 1989a; Osmond 1990; 1991). As demonstrated in Figure 4, the B-cell compartment of the bone marrow were divided as: Pro-B cells subdivided to early pro-B (TdT^+B220^-), intermediate pro-B (TdT^+B220^+) and late pro-B (TdT^-B220^+); pre-B cells exhibiting cytoplasmic μ heavy chain and lacking TdT subdivided to large pre-B and small pre-B cells; and finally B lymphocytes expressing surface IgM.

Osmond (1990, 1991) in his kinetic studies demonstrated that the early B-cell compartment including pro-B and pre-B cells undergo a series of mitoses, at least once at each phenotypic stage of development with the exception of small pre-B cells that show much lower turnover rate (Figure 4). As shown in Figure 4, the turn over rate of large pre-B cells followed by smaller turn over rate of small pre-B cells indicated a dramatic change in this transition state. If this significant change is translated into less survival of small pre-B cells ,as it is suggested by Osmond (1993), then, there is a significant cell loss at this stage of B-cell development. This substantial cell death has been also reported by other investigators

(Jacobsen *et al.*, 1994; Osmond *et al.*, 1994). It is suggested that this cell loss is mainly due to defective Ig gene rearrangements (Jacobsen *et al.*, 1994), which has also been suggested to occur among cells of late pro-B cell subset ($TdT^{-}B220^{+}\mu^{-}$) which may have undergone nonproductive gene rearrangements (Rolink and Melchers 1993; Rolink *et al.*, 1994). *In vivo* studies have suggested that much of the loss occurs via apoptotic cell death followed by macrophage mediated phagocytic elimination of these cells (Jacobson *et al.*, 1994; Osmond *et al.*, 1994). This cell loss provides a control mechanism to eliminate cells with nonfunctional gene rearrangement and to regulate cell numbers entering the circulation. It is estimated that out of $5x10^{7}$ cells generated from B-cell progenitors in the BM of adult mouse, only $2\text{-}5x10^{6}$ cells are released to the peripheral pool each day (Rolink and Melchers, 1993). Indeed, this dramatic cell loss at the pre-B stage of development is highly significant for the study presented in this dissertation since it suggests the high susceptibility of these cells to apoptotic cell death which might play a regulatory role in lymphopoietic processes. The question of whether or not zinc deficiency and the subsequent increased endogenous glucocorticoids would adversely affect this population that is highly programmed to die will be addressed in Chapters two and three of this dissertation.

As previously mentioned, early stages of B-cell development have been identified via surface expression or elimination of maturation markers, gene recombination and expression, and growth requirements. However, the studies herein have utilized only one aspect of these developmental markers That is, the surface expression of B-cell maturation markers. The availability and the specificity of monoclonal antibodies (mAbs) against stage specific surface markers on B-lymphocytes and the flow cytometric determination of different lymphocyte

subpopulations were efficient tools for the evaluation of the BM B-cell genesis. Thus, a brief review on the structural and functional properties of major early B-cells surface markers, most of which were used in this dissertation, will be given below.

Structural and Functional Properties of Key B-Cell Maturation Markers:

B-lineage cells can be recognized by exclusive expression of the high molecular weight form (220 KDa) of the leukocyte common antigen (L-CA) designated as B220 (CD45R) throughout the stages of B-cell maturation on the surface of all B committed and mature B-cells (Coffman and Weissman, 1981). This transmembrane glycoprotein is composed of composed of elongated, heavily glycosylated exterior domain (~538 residues and less conserved) and a large globular cytoplasmic domain (705 residues and highly conserved) with a 22 amino acids membrane spanning region. Within the cytoplasmic domain there is an internal duplication of about 300 amino acid residues with 33% homology within each duplication (Thomas and Lefrancois, 1988). These regions of duplications has been shown to have protein tyrosine phosphatase (PTPase) activity (Koretzky *et al.*, 1992). It is suggested that this portion of the molecule is involved in the signal transduction pathway by acting on $P59_{fyn}$ and $P56_{lck}$, of the Src-family protein-tyrosine kinases, in T-cells and $P21_{ras}$ in B-cells, activating the signal transduction complex (Guttinger *et al.*, 1992; Rothstein *et al.*, 1993; Kawauchi *et al.*, 1994). This is the molecule that has been selected in this dissertation to identify BM B-lymphocytes, by using the mAb, RA3-6B2. There is no known cross reactivity of this mAb with the L-CA found on T-cells (T-200) (Thomas and Lefrancois, 1988) (see Figure 3).

HSA, Heat stable antigen, is a molecule widely distributed on many cell types with heterogeneous forms of 30-60 KDa depending on the cell type (Wenger *et al.*, 1991). Molecular cloning and sequencing analysis of HSA showed a very short 27-amino acid polypeptide that is anchored to the cell surface through a glycosyl-phosphatidylinositol linkage with extensive N-and O-linked glycosylation (Wenger *et al.*, 1991; 1993). This molecule shows a fluctuating pattern of expression during B-cell differentiation indicating its possible role in B-cell development. HSA is first detectable at low levels on the D-J rearranged ($B220^{+}CD43^{+}$) cells, then reaches the highest levels at the large cycling pro-B to late pre-B stage (Hardy *et al.*, 1991; Ehlish *et al.*, 1993). Immature B cells and newly generated B cells in the spleen all express high levels of HSA. The expression of this molecule is down regulated on most peripheral B-cells, increases upon B-cell activation and becomes low or non-detectable in memory B cells and plasma cells (Allman *et al.*, 1992; 1993) (see Figure 3). HSA is recognized by several antibodies such as 30F1, J11d, M1/69, and 79, all of which have shown similar specificity and staining patterns for HSA molecule (Hardy *et al.*, 1991; Hough *et al.*, 1996; Hahne *et al.*, 1994).

In terms of its function, HSA was first identified as a costimulatory molecule on activated B-cells, dendritic cells and epidermal langerhans cells being essential for the induction of proliferative response in $CD4^{+}$ T-helper cells (Hubbe and Altevogt, 1994; Liu *et al.*, 1992; Enk and Katz, 1994). The second function assigned to HSA is as an adhesion molecule (Sammar *et al.*, 1994). It has been shown that mAb 79 against HSA inhibited the aggregation of lipopolysaccharide (LPS)-activated spleen B cells and induced an increase in intracellular Ca^{++} levels (Hahne *et al.*, 1994). Hough and colleagues in their two most recent

studies evaluated the role of murine HSA in lymphocyte maturation. Using transgenic mice with overexpressed HSA, their data clearly showed perturbation of T and B lymphocyte development. This was indicated by major reductions in both double-positive ($CD4^+CD8^+$) and single-positive ($CD4^+CD8^-$; $CD4^-$ $CD8^+$) thymocytes, profound depletion of pro and pre B-cells especially at the level of IL7-responsive B-cells, and reduced mature peripheral and splenic B-cells with impaired response to LPS stimulation (Hough *et al.*, 1994; 1996). Thus, these observations suggested a regulatory function for HSA throughout the early stages of T and B cell development. Being a general marker for B-lymphocytes and being detected on more than 90% of nucleated BM cells (personal observation), the HSA molecule was not evaluated in phenotypic determination of B-lymhpocytes presented in Chapter four. Nevertheless, this study was able to identify the same B-cell subsets as when HSA would have been included.

CD43 or leukosialin (also known as sialophorin in humans, LY48 or mouse CD43 (S7) in mice, and W3/13 in rats) is a major sialoglycoprotein present on granulocytes, macrophages, T cells, erythroid and B cells at specific stages of development (Gulley *et al.*, 1988; Fukuda, 1991). In mice, this molecule is recognized by rat mAb S7 and has been detected on early B cell progenitors and is lost as these cells differentiate to pre-B and mature B-cells but is upregulated on terminally differentiated plasma cells (Hardy *et al.*, 1991). More recently an additional site of expression for this molecule was found on both mice and human pluripotent hematopoietic stem cells (PHSC) by Moore and his coworkers (1994). These cells (PHSC) upon transfer into SCID (severely combined immunodeficient) mice caused rapid population of BM, spleen and thymus and repopulation of lymphohemopoietic cells in

secondary recipients. The expression of CD43 on only a subset of B-lymphocytes in the BM was significant to this study where the combination of this marker with a predominant B-cell determinant such as B220 could exclusively identify the population of interest, namely progenitor B-cells. This will be addressed in Chapter three of this dissertation.

CD43 molecule has a highly O-glycosylated extracellular domain and a highly conserved transmembrane and intracellular domains within murine and human species (Pallant *et al.*, 1989). The conserved intracellular/transmembrane domains in CD43 have been postulated to play a role in intracellular events such as signal transduction or interaction with cytoskeletal structures (Yonemura *et al.*, 1993). Furthermore, the heavy glycosylation of CD43 extracellular domain is thought to interact with lectin-like receptors on cells (Greaves *et al.*, 1992). In terms of its function, human CD43 has been shown to act as cell adhesion molecule on T cells via binding to intracellular cell adhesion molecule 1 (ICAM-1) on stromal cells (Rosenstein *et al.*, 1991), therefore, suggesting its involvement in cell adhesion and cell proliferation. Most recently, a group of investigators studied the in-vivo effects of disregulated expression of CD43 in B-cell lineage of transgenic mice (generated by microinjection of the infused mouse CD43-IgH chain enhancer gene) (Dragone *et al.*, 1995). These transgenic mice exhibited splenomegaly due to increased number of B-cells, and prolonged survival of their B-cells in culture with decreased apoptosis. Based on these observations they suggested a role for CD43 in delivering signals by itself or by its conjunction with other molecules in the adhesion cascade (i.e., lectin-like receptor on stromal cells) to rescue B-cells from apoptotic death and to promote B-cell expansion and development. Indeed the suggested antiapoptotic role of CD43 molecule in B-lineage cells

would give better understanding for the observed distribution pattern of CD43 bearing early B-lineage cells in the zinc deficient mice which will be presented in Chapter three of this dissertation.

BP-1/6C3 molecule is a 140 KDa cell surface homodimer glycoprotein formed by disulfide linked chains which is identified in mice by the mAb BP-1 and rat mAb 6C3 (Cooper *et al.*, 1986; Wu *et al.*, 1989). This molecule is expressed on early B-lineage cells in BM and in relatively high levels on most neoplastic pre-B cells; pre-B cells in long term bone marrow cultures; certain stromal cell lines; brush borders of the proximal renal tubules and small intestinal enterocytes; and a subpopulation of thymus cortical epithelial cells (Wu *et al.*, 1989; Whitlock *et al.*, 1987; Welch *et al.*, 1990). The broad tissue distribution and the transitional expression of BP1/6C3 on early B-cells support the diverse biological function and the highly ordered regulation of this molecule in B-cell development.

In a sequencing study, BP-1/6C3 was shown to be a member of zinc-metalloprotease family with a highest homology to aminopeptidase N (APN; microsomal aminopeptidase) which acts on peptides with an N-terminal neutral amino acid (Kenny *et al.*, 1987). A few years later, however, another group of investigators demonstrated aminopeptidase A (APA) activity of BP-1/6C3 (Wu *et al.*, 1991). This enzyme catalyzes the removal of N-terminal acidic amino acid (i.e., glutamic and aspartic) residues from peptides. This peptidase activity of BP-1 molecule may be significant in activating or inhibiting the activity of molecules that are involved in cell progression through differentiation pathway. Collectively, the limited expression of BP-1 molecule on early stages of B-lymphopoiesis; its expression on BM-derived stromal cell lines; and the IL-7 (a BM stromal cell cytokine) induced proliferation of

B-cell precursors expressing BP-1, all suggest the important role of this molecule in growth and differentiation of early B-cells. Thus, the study presented in Chapter three utilized this molecule to specifically identify and investigate a small subset of bone marrow early B-cells (late Pro-B) that would have not been possible, otherwise.

T Cell Development in the Thymus:

In contrast to B-lymphocytes that are produced and mature in the BM, the cells committed to differentiate into T-lymphocytes are generated in the BM, but then migrate to the thymus to mature. By analogy to B-cell development in the BM, differentiation of progenitor T-cells is associated with expression of different surface markers as well as rearrangements of the germ-line T-cell receptor (TCR) genes (Pawlowski and Staterz, 1994). The early precursors of T-lymphocytes within the thymus express neither of the major T-cell accessory molecules CD4 and CD8 so are referred to as double-negative (DN) cells. These DN ($CD4^-CD8^-$) thymocytes differentiate into early double-positive (DP) cells, which express low levels of CD4 and CD8 ($CD4^+CD8^+$). This transition in phenotype is associated with extensive proliferation and rearrangement of germ-line TCR α and β gene segments which is very similar to Ig gene rearrangement in B-cells (von Boehmer, 1992; Kruisbeck, 1993; Godfrey *et al.*, 1994). DP thymocytes with non-productive TCR-gene rearrangement will be programmed to die but the productively rearranged $\alpha\beta$ heterodimer of TCR will then be associated with CD3 and will be expressed on the cell surface of DP thymocytes (Hedrick and Eidelman, 1993).

The fate of most developing thymocytes is intrathymic death, either early in the development due to the failure of the TCR molecule to be engaged by self-MHC, or later in

maturation due to self-reactive thymocytes. Through the combination of positive and negative selections, these cells (>95%) will be eliminated via programmed cell death (apoptosis), and the remaining thymocytes (~5%) that are capable of interacting with self-MHC/antigen will survive (Sprent *et al.*, 1988; von Boehmer and Kisielow, 1990; Tough and Sprent, 1994). Differentiation and maturity of thymocytes are thought to be supported by thymic epithelial cells (TECs) through direct cell contact and secretion of thymic hormones (von Boehmer, 1992; Anderson *et al.*, 1994; Coto *et al.*, 1992). A number of thymic hormone-like peptides that influence this maturity have been isolated from the thymus, including thymulin (Bach *et al.*, 1975). This zinc-dependent hormone is thought to play an important role in T-cell differentiation and maturation and subsequent cell-mediated immunity (Coto *et al.*, 1992; Okamato *et al.*, 1993), as *in vitro* addition of thymulin to cultured thymocytes enhanced their response to mitogens (i.e., PHA; ConA) (Saha *et al.*, 1995).

The immature $CD4^+CD8^+TCR^{lo}$ thymocytes that survive the thymic selection develop into TCR^{hi} single-positive $CD4^+$ or $CD8^+$ mature T-cells representing 10-14% or 5-8% of total thymic T-lymphocytes, respectively (Sprent and Webb, 1987; Fowlkers and Pardoll, 1988; van Ewijk, 1991). These mature single positive T-cells ($CD4^+CD8^-TCR^{hi}$/$CD4^-CD8^+TCR^{hi}$) leave the medulla through veins and lymphatic vessels to join the recirculating lymphocyte pool and home to peripheral lymphoid organs (Shortman *et al.*, 1990). In terms of cell numbers, it has been estimated that about $1x10^6$ mature T-cells per day leave the thymus and enter the peripheral blood in young mice (Tough and Sprent, 1994).

Due to the fact that immature thymocytes are highly susceptible to apoptotic death, the presence of thymic atrophy in ZD with preferential involution of the cortex, where

immature thymocytes reside, could suggest of a similar phenomenon. Thus, it was of interest to evaluate the effects of ZD on phenotypic distribution of thymic T-cells, particularly immature DP thymocytes as well as the verification of apoptosis as a mean for elimination of cortical thymocytes manifested as thymic atrophy in ZD. These will be addressed in Chapter Five of this dissertation.

Figure 1: Differentiation and maturation pathways of all major blood cell types from a common hematopoietic stem cell (Ikuta *et al.*, 1992).

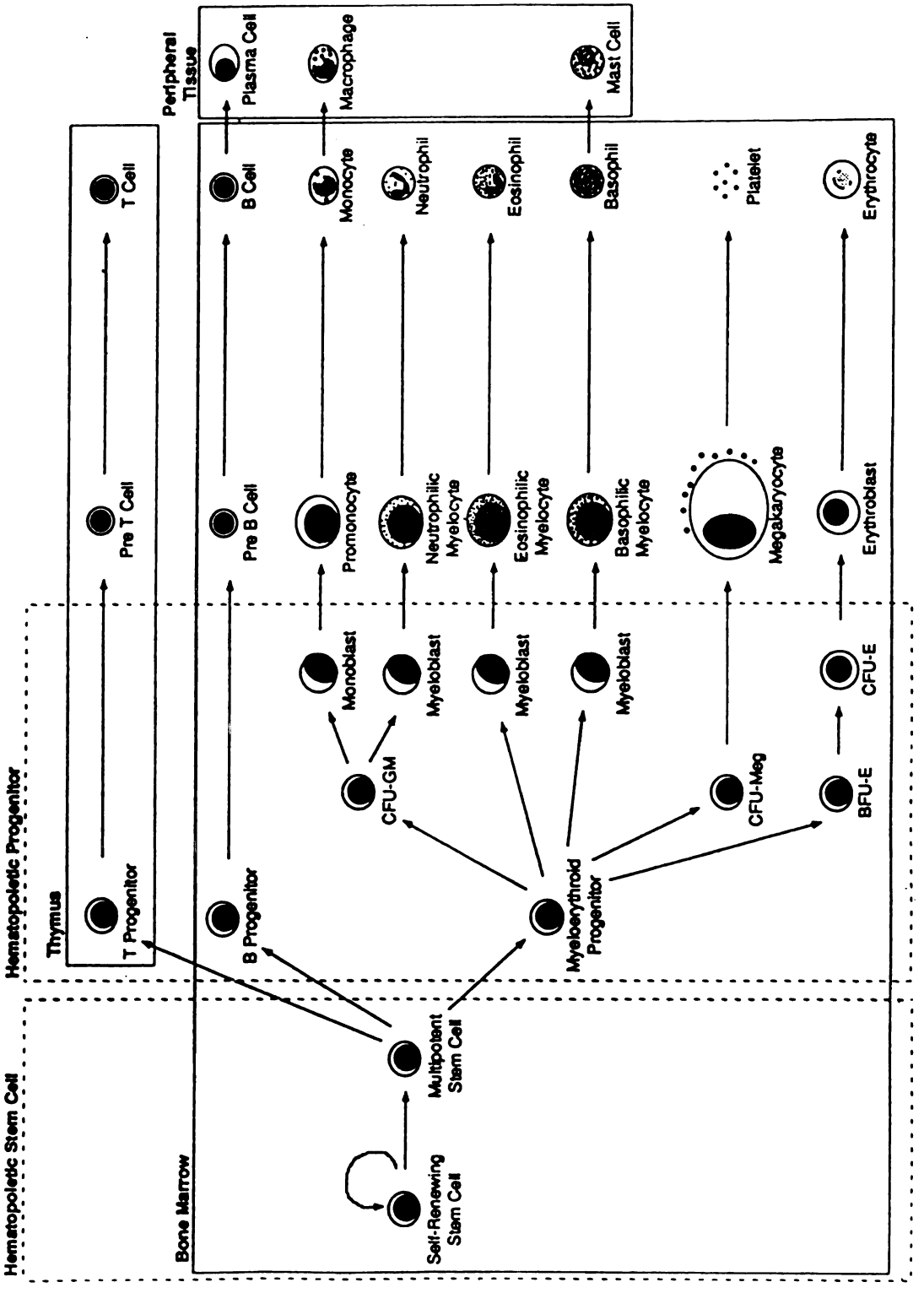

Hematopoietic Stem Cell
Hematopoietic Progenitor
Bone Marrow
Thymus
Peripheral Tissue
Self-Renewing Stem Cell
Multipotent Stem Cell
T Progenitor
B Progenitor
Myeloerythroid Progenitor
Pre T Cell
T Cell
Pre B Cell
B Cell
Plasma Cell
Monoblast
Promonocyte
Monocyte
Macrophage
CFU-GM
Myeloblast
Neutrophilic Myelocyte
Neutrophil
Myeloblast
Eosinophilic Myelocyte
Eosinophil
Myeloblast
Basophilic Myelocyte
Basophil
Mast Cell
CFU-Meg
Megakaryocyte
Platelet
BFU-E
CFU-E
Erythroblast
Erythrocyte

Figure 2: A schematic view of the events required for heavy chain gene rearrangements and generation of μ heavy chain protein (Kuby, 1992).

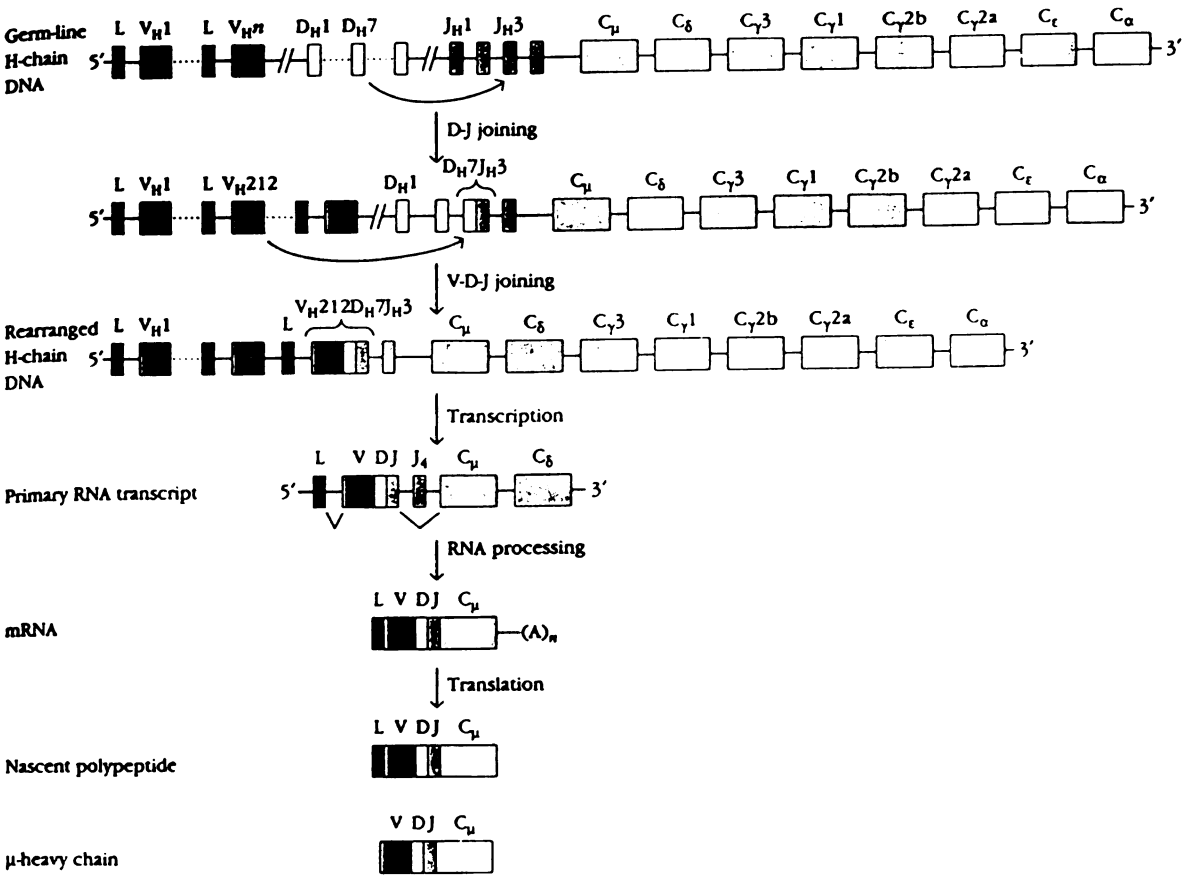

Germ-line H-chain DNA
L VH1 L VHn DH1 DH7 JH1 JH3 Cμ Cδ Cγ3 Cγ1 Cγ2b Cγ2a Cε Cα
5′
3′
D-J joining
L VH1 L VH212 DH1 DH7JH3 Cμ Cδ Cγ3 Cγ1 Cγ2b Cγ2a Cε Cα
V-D-J joining
Rearranged H-chain DNA
L VH1 L VH212DH7JH3 Cμ Cδ Cγ3 Cγ1 Cγ2b Cγ2a Cε Cα
Transcription
Primary RNA transcript
L V DJ J4 Cμ Cδ
RNA processing
mRNA
L V DJ Cμ (A)n
Translation
Nascent polypeptide
L V DJ Cμ
μ-heavy chain
V DJ Cμ

Figure 3: Cell surface and molecular marker expression during the stages of B-cell development in murine bone marrow.

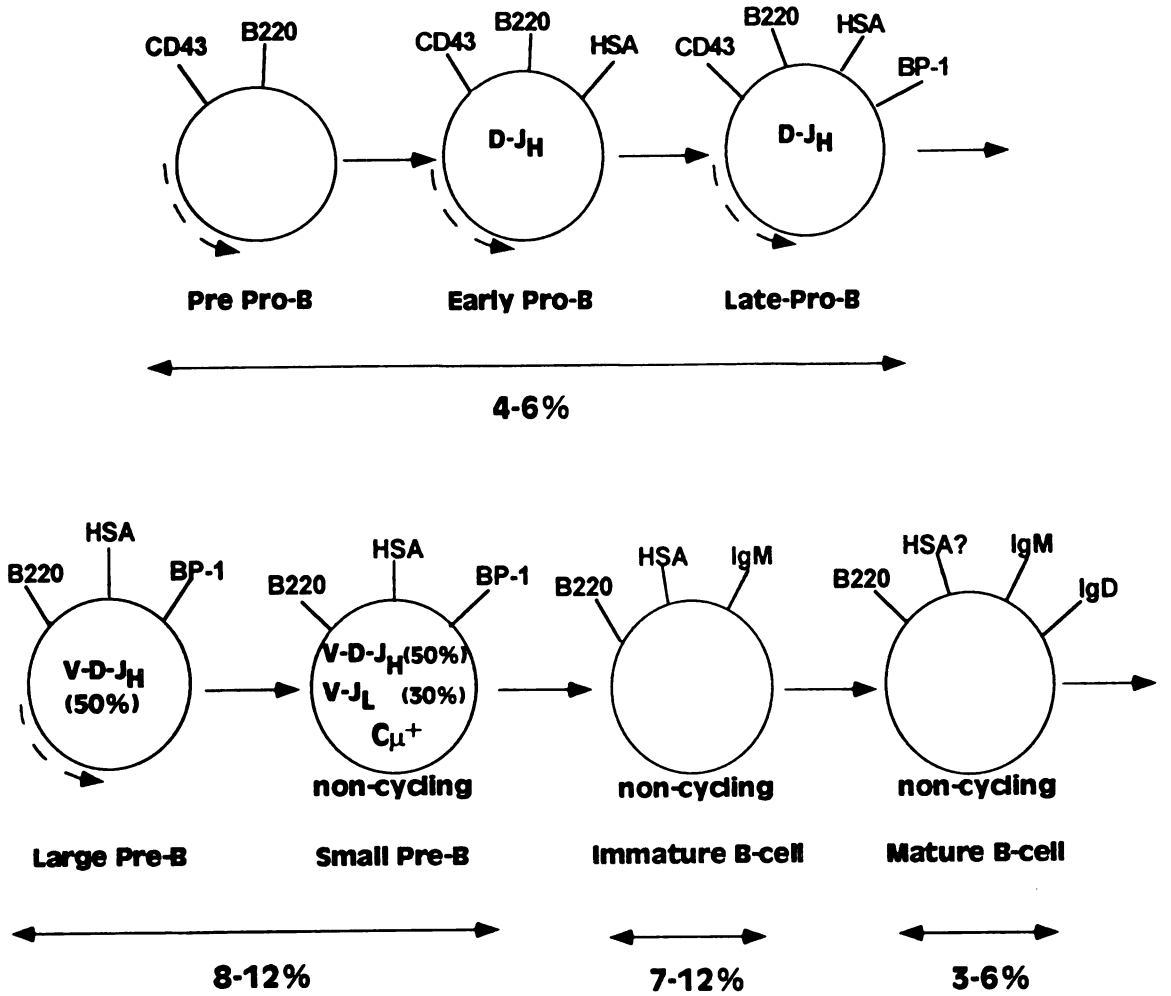
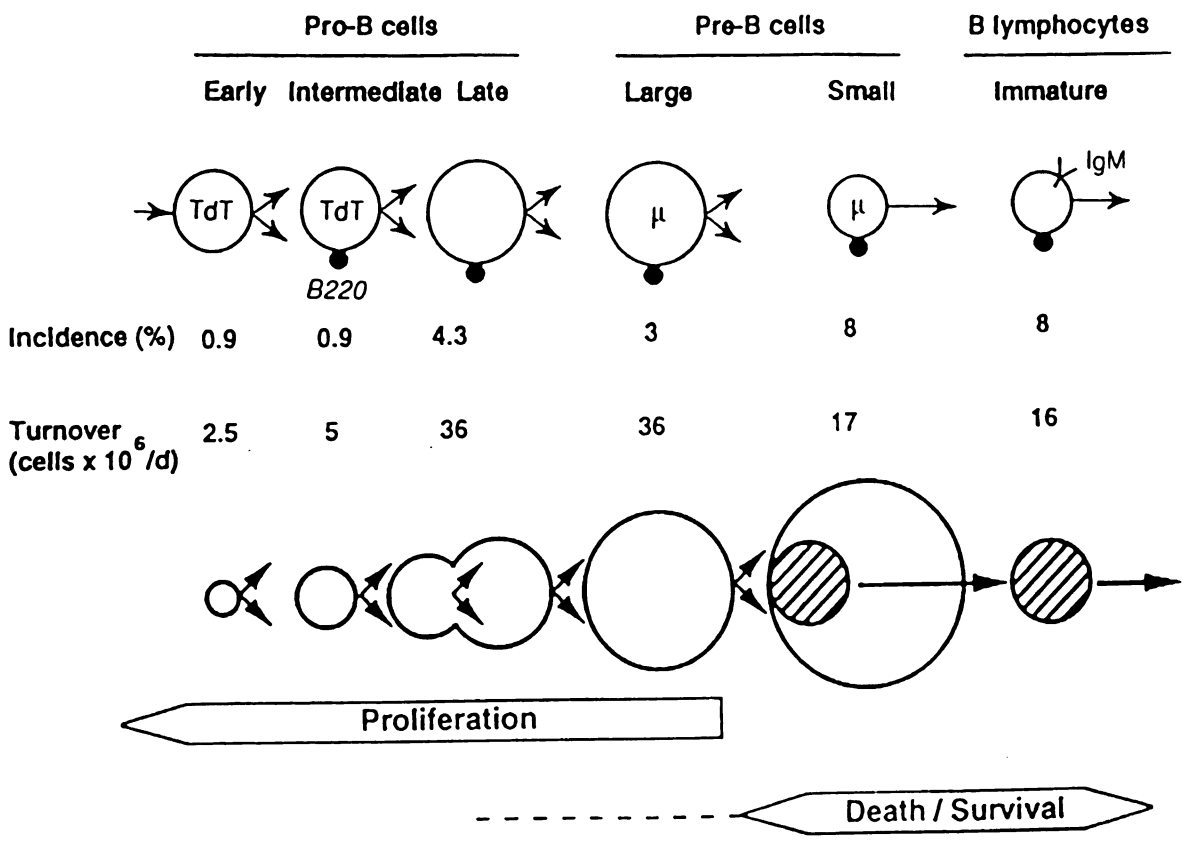

CD43
B220
CD43
B220
HSA
D-J$_H$
CD43
B220
HSA
BP-1
D-J$_H$
Pre Pro-B
Early Pro-B
Late-Pro-B
4-6%
HSA
B220
BP-1
V-D-J$_H$ (50%)
HSA
B220
BP-1
V-D-J$_H$(50%)
V-J$_L$ (30%)
Cμ^+
non-cycling
HSA
IgM
B220
non-cycling
HSA?
IgM
IgD
B220
non-cycling
Large Pre-B
Small Pre-B
Immature B-cell
Mature B-cell
8-12%
7-12%
3-6%

Figure 4: Osmond's scheme of B-lymphocyte differentiation and dynamics of B-cell production in murine bone marrow.


Pro-B cells
Pre-B cells
B lymphocytes
Early
Intermediate
Late
Large
Small
Immature
TdT
TdT
B220
μ
μ
IgM
Incidence (%) 0.9 0.9 4.3 3 8 8
Turnover (cells x 10^6/d) 2.5 5 36 36 17 16
Proliferation
Death / Survival

LITERATURE REVIEW

SECTION IV: Effects of Zinc Deficiency on Stress Axis;

Role of Glucocorticoids on Immune System

Effects of zinc deficiency on stress axis:

Selye in his book, *The Stress of Life* (1956), clearly demonstrated that stress from a variety of sources caused adrenal enlargement, increased serum glucocorticoids (GCs) and thymus atrophy and that stress-induced atrophy was much diminished in adrenalectomized rats. These observations led to the speculation that the activation of hypothalamus-pituitary-adrenal axis (HPA axis; stress axis) played as an important link between the neuroendocrine and immune systems during stress. Since then, chronic elevation of endogenous GCs followed by downsizing of the immune system has been documented in various stress related conditions including: burn and trauma (Maldonado *et al.*, 1991); infection (Hermann *et al.*, 1994); post surgery ; and malnutrition (Barone *et al.*, 1993; Fraker *et al.*, 1995). It has been noted that zinc deficiency (ZD) activates the stress axis and leads to the chronic elevation of GCs in the circulation. In fact, there are studies suggesting the role of elevated endogenous GCs in the induction of immunological alterations such as thymic atrophy, and lymphopenia observed in ZD (Quarterman and Humphries, 1979; DePasquale-Jardieu and Fraker, 1979; 1980; Fraker *et al.*, 1995). In addition to trace element nutritional deficiency, other forms of malnutrition, such as protein-energy-malnutrition, which resemble ZD in terms of the impaired immune system also result in elevation of GCs followed by profound thymic atrophy, and lymphopenia (Becker, 1983; Barone *et al.*, 1993; Kuvibidila *et al.*, 1993). Thus it appears that the elevation of glucocorticoids is common to all the conditions in which downsizing of the immune system has been reported.

A series of early studies by Quarterman (1972; 1974; Quarterman and Humphries, 1979) demonstrated thymic atrophy and enlargement of the adrenal glands with concomitant

increase in cholesterol (precursor of glucocorticoids) content in ZD rats. These observations were followed by a significant increase in plasma corticosterone (CS) level (about 2-fold) in ZD rats compared to the control group. Interestingly, when ZD rats were given a large amounts of zinc (~10 mg zinc sulfate per day in drinking water), an increase in thymus weight and a decrease in plasma CS concentration to levels detected in control zinc adequate (ZA) rats were observed. In a similar study, increased pituitary and adrenal weights, accompanied by hypersecretion of adrenocorticotropin hormone (ACTH; released from pituitary gland), and adrenal corticoid hormones were reported in ZD albino rats (Macapinlac *et al.*, 1966). These observations led to speculation that stress-induced elevation of GCs was playing a major role in suppression of immunity. A few years later this hypothesis was investigated by DePasquale-Jardieu and Fraker (1979; 1980). They noted that ZD mice exhibited thymic atrophy with a preferential involution of the thymic cortex as well as significantly elevated GC levels (3-fold higher than control mice). Furthermore, a concomitant reduction in T-dependent antibody mediated response and rise in CS concentration suggested a role for GCs in the suppression of immune response (DePasquale-Jardieu and Fraker, 1979). If CS played a key role in impaired immunity, specifically thymic involution in ZD, then the elimination of this hormone should be able to block such immunological defects. This was tested by adrenalectomizing mice and removing CS from the circulation (DePasquale-Jardieu and Fraker, 1980). The results indicated complete thymic protection in adrenalectomized ZD mice, whereas the sham-operated ZD mice showed a significant thymic involution. Similar results were also observed in adrenalectomized ZD rats by another investigator (Quarterman and Humphries, 1979). When DePasquale-Jardieu and Fraker evaluated the T-cell helper-

mediated antibody response of zinc dietary mice, a significant drop (50%) in the response in the ZD mice was noted before any elevation of serum CS, possibly due to other factors (eg., nutritional deficiency). However, another reduction (20%) in antibody response was noted in just sham-operated ZD mice who exhibited significant elevation of GCs (~7 fold increase) in their serum (DePasquale-Jardieu and Fraker, 1980). These findings indicated the impaired T-helper function and the sensitivity of cortical thymocytes (immature T-cells) to the elevated levels of GCs manifested as thymic involution since adrenalectomized ZD mice showed complete thymic protection. Furthermore, it suggested a combination of both suboptimal zinc intake and elevated CS in the suppression of immune response.

Thus the use of adrenalectomy was able to begin to clarify the role of elevated GC levels during ZD in thymic involution and elimination of immature cortical thymocytes as well as the impairment of cell-mediated immunity due to T-helper cell dysfunctions. In fact the sensitivity of immature T-cells to GCs followed by glucocorticoid-induced apoptotic death in this population is a well established phenomenon (McConkey *et al.* 1989; Cohen and Duck, 1992; Sun *et al.*, 1992; Brown *et al.*, 1993). The GC sensitivity was not just restricted to T-cells, as B-cells were also shown to be affected. In a CS pellet implantation system in young adult mice delivering CS analogous to levels detected in ZD, a significant depletion in BM total B-cells and immature B-cells (75% and 25% respectively) were noted within five days (Garvy *et al.*, 1993a). However, mature B-cells were moderately increased. This suggested the preferential elimination of immature B-cells by CS and the resistance of more mature B-

cell population. Detailed studies on distribution of BM B-lymphocytes in ZD mice will be presented in Chapters two and three.

Knowing that both immature T and B cells are highly sensitive to the elevated GCs and adrenalectomy provides protection against thymic involution, it was of interest to examine the status of B-lymphocytes in adrenalectomized ZD mice. Chapter four will thoroughly address the role of chronic elevation of GCs in ZD on BM B-cell development, thus, identifying a more clear role for GCs in nutritional deficiencies.

General Characteristics of Glucocorticoids:

Glucocorticoids are in a class of major steroid hormones released from zona fasciculata of the adrenal cortex. These hormones have been shown to have a wide range of effects on components of the immune system and inflammatory responses in both humans and animals (DePasquale-Jardieu and Fraker, 1979; 1980; Garvy *et al.*, 1993a; 1993b; Flaherty *et al.*, 1993; Adcock *et al.*, 1995; Marx, 1995). In terms of their synthesis, cholesterol is the known biosynthetic source of all steroid hormones, including GCs (Miller, 1988). Adrenocortical cells have large numbers of receptors that mediate the uptake of low-density lipoprotein (LDL), the predominant form of cholesterol, into the cells. The cholesterol is then enzymatically converted to pregnenolone via cholesterol desmolase. Dehydrogenation of pregnenolone yields progesterone which is the precursor to all steroid hormones (Bolander, 1994; Miller, 1988; Hadley, 1992; Rudney and Sexton, 1986). The biosynthelic pathways of steroid hormones are summarized in Figure 1.

Production and release of GCs by the adrenal cortex is primarily under control of ACTH released from the anterior pituitary gland. In turn, the release of ACTH is regulated

by a variety of neurohypophysical peptides produced by hypothalamic neurons. One of the main regulating peptides is corticotropin-releasing factor (CRF) which is released at neuronal endings and transported via the portal vein to the anterior pituitary where it stimulates the synthesis and secretion of ACTH (Figure 2) (Berczi, 1994; Bolander, 1994; Hadley, 1992). The effects of CRF and ACTH are mediated by activation of adenylate cyclase and the cyclic AMP-dependent protein kinase (Hadley, 1992). Briefly, ACTH interacts with plasma membrane receptors of the zona fasciculata cells of adrenal cortex and activates adenylate cyclase. This activation results in increased cAMP levels and subsequent activation of one or more protein kinases. The active phosphorylated protein will activate a cholesterol ester hydrolase to convert sequestered cholesterol into free cholesterol available for steroidogenesis (Hadley, 1992).

Cortisol (hydrocortisone; 11β, 17α, 21-trihydroxy-pregn-4-ene 3, 20, dione) and corticosterone (17α, 21-dihydroxy-pregn-4-ene 3, 11, 21 trione) are the main GCs secreted by the adrenal cortex. However, the relative amounts of these hormones that are generated depend on the species. For example, in man, dog, and monkey, cortisol secretion predominates, whereas in rats, mice and rabbits, corticosterone is the main secretory GC (Simpson and Waterman, 1988). The rate of secretion of cortisol in normal human subject under optimal conditions is about 20-30 mg/day. However, the rate is not steady and exhibits circadian rhythmic, being relatively high in the early morning hours, declining during the day, and reaching a minimum during the evening (Smith *et al.*, 1981; Hadley, 1992). Being nocturnal, the rodents exhibit the reverse diurnal cycle, with the highest CS concentrations in the evening (20 mg/dl) and the lowest in the early morning. With this in mind and due to

the fact that chronic elevation of GCs accompanies ZD, care was taken in this work to collect all blood samples required for CS assay during early morning, 8-9 AM, at the lowest concentration time point in the diurnal cycle for rodents.

In terms of GC transport system, plasma is the main route of GC transport to target tissues. In the plasma, 90% or more of the cortisol and CS is reversibly bound to two main proteins, namely corticosteroid-binding globulin (CBG; transcortin) and albumin (Ballard, 1979; Siiteri *et al.*, 1982; Baxter and Tyrell, 1987). The CBG has been identified as a member of the serine protease inhibitor (SEPRIN) superfamily, with a molecular weight between 50,000-60,000 daltons in most species (Hammond *et al.*, 1987; Nyberg *et al.*, 1990). This protein has high affinity but low total binding capacity for GCs, whereas albumin has low affinity, but relatively large binding capacity. The remaining fraction of GCs in plasma comprises the pool of nonprotein bound or "free" steroid that is generally assumed to be biologically active (Brien, 1981; Siiteri *et al.*, 1982; Vermeulen, 1986). The free hormone model of steroid action has been defined by Lan *et al.*, (1984) as: "only those steroids which are not bound (eg., free) to CBG in plasma are available to diffuse out of the capillary bed into the interstitial space, and go across cell membranes to initiate hormonal effects". In fact in an early study by Slaunwhite *et al.*, (1962), they showed that injection of cortisol alone increased liver glycogen in adrenalectomized mice, whereas injection of cortisol-CBG complex caused no increase, thus demonstrating that CBG-bound cortisol is biologically inactive.

The free hormone model was further supported by the work of Faict *et al.*, (1985). They investigated whether transcortin (CBG) modulates the in vitro effects of cortisol on the

proliferation of human peripheral blood mononuclear cells (PBMC) stimulated by different mitogens. Doses of cortisol in Physiological range (10-1000 nM) strongly inhibited the proliferation of PBMC (80% lymphocytes, 20% monocytes) stimulated by the mAb OKT3, a mitogen specific for T-lymphocytes, and by phytohaemagglutinin (PHA). However, a smaller degree of inhibition was observed with pokeweed mitogen (PWM). As expected, CBG alone had no influence on the proliferation of stimulated PBMC. However, addition of pure cortisol-free transcortin to the cultures significantly reduced the effects of cortisol. It was, thus, concluded that when evaluating the effects of GCs on lymphoid tissues, only the free steroid level rather than the total steroid concentrations should be considered. In fact, this was considered in the study presented in Chapter five in which the amount equall to the estimated biologically active free CS level as opposed to total concentration of CS detected in ZD mice was added to the culture system.

Structure and Function of Glucocorticoid Receptor:

The action of steroid hormones including GC is primarily mediated via binding to a cytoplasmic receptor followed by nucleus translocation and binding of the hormone-receptor complex to the specific DNA sequence of the target cell genome. Briefly, the hormone initially interacts with the cytoplasmic receptor in the target cell, thereby inducing a change in the receptor which is followed by dissociation of the receptor-associated protein complex and exposure of the DNA binding domain. The activated receptor-ligand complex translocates toward the nucleus via the cytoskeleton transport system, and into the nucleus via nuclear pores, where it binds to a consensus binding sequence of target genes. The binding of the receptor-steroid complexes to DNA initiates changes in gene expression that

are translated into the cellular response to GC (Gustafsson *et al.*, 1987; Distelhorst, 1989; Meisfield, 1990).

The glucocorticoid receptor (GR) is a member of the steroid receptor superfamily, which is found in all mammalian tissues but at different levels (Pratt, 1993). The function of GR as transcriptional activator is uniquely hormone dependent, since its nuclear translocation and transcriptional activation only occurs upon ligand binding (Gustafsson *et al.*, 1987; Burnstein and Cidlowski, 1989; Godowski and Picard, 1989; Smith and Toft, 1993). The structure of GR is composed of a ligand binding domain at the C-terminal, a DNA binding domain in the center and a modulating domain at the N-terminal (Figure 3). The DNA binding domain is composed of two zinc-finger structures which have been proposed to interact with the hormone responsive elements (HRE; GRE in case of glucocorticoids) on DNA and regulate transcription of the gene downstream. These zinc atoms are located in a cysteine-rich region of the DNA binding domain, and are each tetrahedrally bound to 4 cysteine residues, forming zinc finger structures (Gustafsson *et al.*, 1987; Luisi *et al.*, 1991; Hutchison *et al.*, 1992). Using point mutations, the presence of zinc fingers in the DNA binding domain has been shown to confer the specificity of receptor binding to GREs (Archer *et al.*, 1990). The GRE is a 15 base-pair, partially palindromic sequence that consist of two hexameric half-sites separated by three bases (Beato, 1989). Based on sequence analysis, a consensus binding sequence, 5′-AGAACAnnnTGTTCT-3′ (where n can be any nucleotide) located within the 5′-flanking regions of the promoter of the targeted genes was proposed (Beato, 1989; Gustaffson *et al.*, 1990). In nature, the sequence of the half-sites of GREs may vary considerably, but the spacing between the half-sites is always three bases. The

palindromic nature of GREs suggest that the receptor binds its targets as dimers. The crystallographic analysis (Luisi *et al.*, 1991) or the electrophoretic mobility studies (Alroy and Freedman, 1992) have shown that two monomers of the GR DNA-binding domain bind to the DNA target site face to face, making extensive protein-protein contacts. The binding of the first monomer increases the affinity of the second monomer by two orders of magnitude (Hard *et al.*, 1990). Regions required for dimerization are located in both the hormone binding and DNA binding domains (Dahlman-Wright *et al.*, 1993) (see Figure 3).

Early studies on isolation and characterization of steroid receptors led to the isolation of two steroid binding forms: a large 8-9S form and a smaller 4S form (Pratt, 1987). The 8-9S receptor form was found predominantly in the cytoplasm, whereas the 4S form was detected in the nucleus. Furthermore, stimulation of hormone decreased the amount of 8-9S form in the cytoplasm and increased the amount of 4S form in the nucleus, indicating hormone induced transformation and nuclear translocation of the receptor (LaFond *et al.*, 1988). The untransformed cytosolic receptor is predominantly associated with a 90KDa heat shock protein (hsp90) which is dissociated upon ligand binding and receptor transformation to the 4S form (Sanchez *et al.*, 1986; Housley *et al.*, 1990). Further studies have established that hsp90 association is required for steroid binding to the GR, whereas dissociation of hsp90 precedes the DNA binding of receptor-steroid complex (Meshinchi *et al.*, 1990; Hutchiso *et al.*, 1992). Furthermore, hsp90 is thought to stabilize the receptor, and prevent it from binding to DNA by masking the domains required for receptor localization and DNA binding (Housley *et al.*, 1990; Pratt, 1993). Sequence studies and insertional/deletional mutations of GR have identified a 20 amino acid sequence at the N-terminal region of the steroid binding

domain for hsp90 association (Pratt *et al.*, 1988; Housley *et al.*, 1990). Thus the proximity of hsp90 binding domain to the steroid binding, DNA binding, and nuclear localization domains support the hsp90 dissociation upon ligand binding and disposing the domains necessary for DNA binding.

In addition to the association of hsp90 as the predominant non-hormone binding protein with GR, there are also a number of proteins associated with the untransformed GR. Among them is hsp70 that is present in chinese hamster ovary cells (CHO) that overexpress the mouse GR and for some unknown reasons is entirely nuclear in hormone free CHO cells (Sanchez *et al.*, 1990a; 1990b). It is important to note that as opposed to hsp70 association and localization of mouse GR in CHO cells, the mouse GR in L cells is cytoplasmic and not associated with hsp70 (Snachez *et al.*, 1990b). This has caused an speculation that the presence of receptor-associated hsp70 is in some way related to the arrival of steroid receptors in the nucleus, thereby serving as a molecular chaperon (Sanchez *et al.*, 1990b; Pratt and Scherrer, 1994). It is thought that hsp70 binds to hydrophobic regions of proteins to facilitate their unfolding to cross organelle's membranes (Rothman, 1989). The association of GR with a complex containing hsp70 could play a role in maintaining steroid receptors in an unfolded state for passage through the nuclear membrane (Sanchez *et al.*, 1990b; Pratt *et al.*, 1992). In this respect Shi and Thomas (1992) have shown an hsp70 requirement for transport of nucleoplasmin into Hela cell nuclei.

Besides the association of hsp90 and hsp70 with GR, a number of other proteins such as hsp59 (also known as hsp56, hsp60), p50, p23, and p14 has been reported to occur as a part of heteromeric structure of untransformed GR (Pratt, 1992; Pratt, 1993; Lebeau *et al.*,

1994) (Figure 4). In fact, coimmunoadsorption studies with GR (Bresnick *et al.*, 1990; Sanchez *et al.*, 1990b; Pratt, 1990) suggest that the receptor-hsp90 complex is a core unit derived from a larger heteromeric complex that also contains hsp70, hsp56, and some other proteins. The heat shock protein heterocomplex exists in cytosol independent of steroid receptors (Sanchez *et al.*, 1990a; Tai *et al.*, 1992) and the three heat shock proteins in the complex are thought to be involved in protein folding/unfolding and protein trafficking in the cell (Pratt *et al.*, 1992). This heterocomplex has been suggested to function as a transport particle, thus termed a "Transportosome" to which the steroid receptor remain attached while they undergo trafficking via cytoskeleton-mediated transport within the cell (Pratt, 1992).

Following steroid binding, receptor dissociation from hsp90 with its simultaneous transformation to DNA binding state, and cytoskeletal-mediated transport, the receptor must translocate across the nuclear membrane to access to the nuclear chromatin via nuclear pores. Nuclear proteins larger than relative molecular mass of ~40,000 appear to require a nuclear localization signal (NLS) for passage through the nuclear pores (Pratt and Scherrer, 1994). Picard and Yamamoto (1987) have identified two nuclear localization signals, NL1 and NL2, in the COOH-terminal half of the GR. NL1 maps to a short segment at the COOH-terminal side of the DNA binding domain, whereas NL2 is located within the hormone binding of the receptor. Both NLS are hormone dependent, accounting for cytoplasmic location of the GR in the absence of hormone (Picard and Yamamoto, 1987). Thus, it appears that hsp90 caps both DNA binding and nuclear localization domains, which are uncapped upon ligand binding, thus allowing the progression of both processes. The transformed receptor would then move along the cytoskeletal (microtubules, microfilaments) pathway toward the nuclear envelope

and translocate across the nuclear membrane through nuclear pores using NLS to reach the nuclear chromatin. These receptors can be exported out of the nucleus to be recycled (Madan and DeFranco, 1993). Subsequent binding of the transformed active receptor to the GRE on DNA, would then either stimulate or inhibits transcription of the targeted gene (Pratt *et al.*, 1989; Meisfield, 1990; Pratt, 1993).

Two receptor subtypes for adrenal steroids have been characterized: type I receptors, or mineralocorticoid receptors, and type II receptors known as glucocorticoid receptors. Type I receptors have a higher affinity for naturally occurring adrenal steroids (cortisol in men and corticosterone in rodents) (Beaumont and Fanestil, 1983; Spencer *et al.*, 1991). On the contrary, type II receptors have a higher affinity for synthetic glucocorticoid, dexamethasone (Reul and deKloet, 1985; Sutanto and deKloet, 1987; Reul *et al.*, 1987). Both of these receptors have been identified in immune cells, however, with considerable expression variation among immune tissues (Lowy, 1989; Miller *et al.*, 1990; Spencer *et al.*, 1991). Thymus has been shown to express the highest type II receptor concentration in the body, whereas spleen exhibited both type I and type II receptors (Lowy, 1989; Miller *et al.*, 1990). This pattern of receptor expression is suggestive of the different responses or different sensitivity of various immune compartments to the glucocorticoid hormones (Miller *et al.*, 1990; Miller *et al.*, 1994; Spencer *et al.*, 1993).

Effects of Glucocorticoids on Lymphocytes:

Glucocorticoid hormones have wide-ranging effects on the immune system (Cupps and Fauci, 1982). At pharmacological levels, these hormones exert anti-inflammatory and immunosuppressive effects, whereas at physiological levels they play important

immunoregulatory roles (Munck *et al.*, 1984; Flaherty *et al.*, 1993; Adcok *et al.*, 1995; Marx, 1995; Auphan *et al.*, 1995).

In an early study by Fauci and Dale (1974), the *in vivo* effects of hydrocortisone (cortisol) on subpopulations of lymphoid cells in human peripheral blood were investigated. A single intravenous injection of either 100 mg or 400 mg of hydrocortisone showed a profound decrease in absolute numbers of circulating lymphocytes and monocytes between 4-6 hours after either concentration of hydrocortisone. However, the counts returned to normal by 24 hours. The depletion of lymphocytes from the circulation was selective in that there was a greater decrease in the number of thymus derived T-cells than B-cells, which returned to baseline by 24 hours after hydrocortisone injection. The *in vitro* response to PHA was relatively unaffected, while responses to concanavalin A (Con A), PWM (at high hydrocortisone concentrations=400 mg), and *in vitro* responses to antigens (streptokinase-streptodornase and tetanus toxoid) were significantly diminished. This selective depletion of monocytes and lymphocytes was suggested to be the result of redistribution of these cells out of the circulation into the other body compartments. However, this proved not to be the case in studies herein, which will demonstrate the selective sensitivity of different immune components to the GCs resulting in their elimination or survival rather than their redistribution.

The susceptibility of lymphocytes to GCs was further demonstrated by the study of Miller *et al.*, (1991). In this study they showed that dexamethasone (Dex) treatment (0.3 to 10 μg/hr) had a significant effect on T-cell proliferation where a stepwise increase in the Dex concentration added to the drinking water was associated with a stepwise decrease in the

Con-A induced splenocyte proliferative response. To ascertain that the observed effects were indeed due to the GCs, these investigators initiated a multidimentionals study. This study examined the effects of GCs and the GR agonist on the number and percentage of immune cells in the peripheral blood and spleen of rats (Miller *et al.*, 1994). Implanting corticosterone in adrenalectomized and sham-operated rats for seven days, they demonstrated a significant increase (>50%) in the neutrophil population and a substantial depletion of all lymphocyte subsets including T-cells (helper and suppressor), B-cells, natural killer cells, as well as monocytes in the peripheral blood and the spleen. In addition, the effects of RU28362, a potent GR agonist, on immune cell distribution in the peripheral blood was examined. Implanting osmotic minipumps which delivered 1, 4, and 10 μg/hr of RU28362 resulted in a significant decrease in lymphocyte numbers with concomitant increase in neutrophil population in a dose-dependent manner. Similarly, the high concentration of RU28362 (10 μg/hr) significantly reduced the spleen cellularity, decreased the absolute numbers and the percentage of all lymphocyte subsets, with B-cells exhibiting the greatest decline (>80%), and a substantial increase in the percentage of neutrophils. Consistent with the depletion of B-cells in RU28362 treated rats, a significant increase in B-cell population of peripheral blood in non-treated adrenalectomized animals compared to sham adrenalectomized and RU28362 treated rats was observed. These findings demonstrated the potent effects of chronic delivery of both naturally occurring GCs, corticosterone, and the GR agonist, RU28362, on the distribution of immune cells mainly manifested as lymphopenia and neutrophilia, as adrenalectomy blocked the effects. In this regard, studies presented in Chapter Two and Four will demonstrate the pattern of BM B-lymphocyte development in the presence of

elevated endogenous CS (ZD mice) and in the absence of CS (ZD adrenalectomized mice) in zinc dietary studies, respectively.

The sensitivity of lymphocytes, particularly T-cells, to exposure to GCs was emphasized in a recent study by Flaherty *et al.*, (1993). In this investigation they evaluated the effects of continuous CS administration on peripheral blood lymphocytes of Fischer 344 rats. This was accomplished by subcutaneous pellet implantation releasing CS (0.07, 0.48, and 4.8 mg/day) over a 21-day period. As expected, a significant decrease in total lymphocytes with reductions in the absolute numbers of the T-helper, T-cytotoxic and B-cells was observed. Histopathological examination of animals treated with the high dose CS, for seven days, revealed involution of the thymus with diminished thymic lymphocytes. Due to the high sensitivity of T-cells to GCs, it was important to relate the distribution and the susceptibility of thymic T-lymphocyte subpopulations to the effects of ZD, which is accompanied by thymic atrophy and elevated levels of GCs. This will be addressed in Chapter Five.

Besides the immunosuppressive effects of pharmacological doses of synthetic GCs, stress induced alteration of immune cell distribution due to endogenous GCs has been also reported recently. Dhabhar *et al.*, (1995) demonstrated that restraining rats for up to 2 hours caused a rapid and significant increase in plasma corticosterone levels. This elevation was accompanied by a significant decrease in numbers and percentages of lymphocytes (T-cells, B-cells, NK cells) and monocytes and an increase in numbers and percentages of neutrophils in the peripheral blood. The observed alteration in immune cells was related to the high concentration of corticosterone, since adrenalectomy significantly reduced the depletion of

immune cells. Furthermore, administration of corticosterone to adrenalectomized rats resulted in the same immunological alteration as those observed in stress-induced intact rats.

In thermal injury which results in secretion of elevated levels of GCs, depression of host defense system was observed (Organ *et. al.*, 1989; Calvano *et al.*, 1987; 1988). In this regard, Calvano and his co-workers studied 10 thermally injured human subjects over time for both percentages and absolute numbers of peripheral blood lymphocytes. Their results showed a significant reduction in $CD3^+$ lymphocytes percentage in the early post burn period, with a concomitant decline in $CD4^+$ T-cell subset. The percentage of $CD8^+$ T-cells did not change significantly at any time post burn. The change in T-lymphocyte subsets caused a general lymphopenia on day four following the injury. A previous work from the same laboratory (Calvano *et al.*, 1987) clearly demonstrated the role of GCs in the change of lymphocyte distribution in burn patients, by comparing these patients with healthy individuals infused with hydrocortisone for 6 hours. Both groups showed significant lymphopenia, monocytopenia and granulocytosis. Additionally, there was a substantial decrease in percentage of $CD4^+$ T-cells with no significant change in the percentage of $CD8^+$ cells.

Parallel to these studies, the immunosuppressive effects of GCs on cells of the immune system in protein-calorie-malnutrition (Becker, 1983; Barone *et al.*, 1993) have revealed close similarity to those observed in other stress induced conditions (eg., ZD, trauma and burn patients) (DePasquale-Jardieu and Fraker, 1980; Organ *et al.*, 1989; Maldonado *et al.*, 1991; Fraker *et al.*, 1995). Recent studies on the evaluation of the role of elevated serum CS observed in protein malnutrition demonstrated a substantial sensitivity of T-lymphocytes, particularly immature $CD4^+CD8^+$ and mature $CD4^+CD8^-$subsets along with impaired

macrophage function in mice fed protein deficient (PD) diet (Barone *et al.*, 1993; Hill *et al.*, 1995). Blocking the stress CS response with adrenalectomy or using RU486 to block CS receptor prevented the impairment of macrophage function (Hill *et al.*, 1995). Furthermore, administration of CS via a subcutaneous pellet implantation reproduced macrophage impairment (Hill *et al.*, 1995) and resulted in severe thymic atrophy and lymphopenia analogous to those of PD mice (Barone *et al.*, 1993). Collectively, These results, once again, strongly support the critical role of glucocorticoids in lymphopenia and selective alteration in immune cell distribution which are observed in ZD.

Interestingly, endogenous levels of GC in the absence of any stimuli has also been shown to control the components of the immune system (DelRey *et al.*, 1984). In this study, normal mice and adrenalectomized mice were evaluated. In normal mice, there was an inverse correlation between endogenous levels of GCs (4-26 mg/dl) and splenic mass, splenic cellularity, and numbers of Ig secreting cells. These observations were reversed in adrenalectomized mice, which showed trace amounts of CS (3-4 mg/dl), confirming the contribution of GCs to the immunosuppression.

Recent studies on the evaluation of the effects of GCs on B-cells indicate that B-lymphocytes in the early stages of their development bear the same sensitivity to GCs as immature T-cells do. A recent study by Voetberg *et al.*, (1994) demonstrated the significant susceptibility of murine lymphocytes, specifically BM B-cells, to the GCs, in this case prednisolone (PD). Ten days of exposure to 2-5 mg PD/ml of plasma via pellet implant in mice resulted in a dramatic depletion (50%) of the circulating lymphocytes. Early B-cells ($B220^+IgM^-$) and immature B-cells ($B220^+IgM^+IgD^-$) showed a significant susceptibility to

PD (3-fold decrease), whereas the mature B-cells ($B220^+IgM^+IgD^+$) were resistant. Furthermore, exposure to PD both *in vivo* and *in vitro* also affected the functional capacity of BM-B cells to the T-cell-independent antigen TNP-LPS (trinitrophenylated-lipopolysaccharide). This was demonstrated by a significant inhibition of plaque-forming cells (PFC) production. However, as expected, the GR antagonist, RU38486, caused 50-70% greater PFC production in PD treated cultures.

Garvy and Fraker (1991) showed a substantial decrease (50%) in *in vitro* responses of BM immature B-cells to TNP-LPS with physiological levels of GCs. This inhibition was more significant (50-80% decrease in plaque forming cells) when dexamethasone, the more potent synthetic GC, was utilized. The specificity of the observed effects by GCs was confirmed when RU38486 counteracted the inhibitory effects of the glucocorticoids. Interestingly, the marked inhibition of plaque formation by B-cells was accompanied by a significant depletion in the proportion of B-cells present in Dex-treated culture. Thus the data clearly indicate the inhibitory effects of glucocorticoid hormones on BM B-cell function via significant reduction in the proportion of immune B-cell population.

A significant study recently presented by Garvy *et al.*, (1993a) revealed a clear picture of the role of GCs on BM B-cell component of the immune system in mice. *In vivo* delivery of levels of GCs analogous to that detected during ZD was shown to down regulate the immune system. Implantation of CS tablets in mice resulted in chronic exposure to the hormone levels normally detected during stress, including ZD (DePasquale-Jardieu and Fraker, 1979). The immunosuppressive effects of the chronic exposure to CS was primarily indicated by the severe thymic atrophy within the first day of pellet implantation. Analysis of

BM B-lineage cells indicated a 70% decrease in total B-cells as well as 40% decrease in Ig^+ B-cells. The early B-cells were completely depleted by day 5 and the cells remaining were shown to be mature B-cells. The results were also accompanied by a decrease in the proportion of B-cells in the S phase of the cell cycle. These observations once again emphasize the down regulation of the immune system by the chronic elevation of GCs as shown by thymic atrophy and depletion of immature lymphocytes. Furthermore, the evaluation of the status of B-cell subsets in the presence of GCs enriches the literature which is heavily focused on T-cell susceptibility to GCs.

Although a substantial investigation has been devoted to the immunosuppressive effects of GCs, the effects of chronic elevation of GCs accompanying ZD on the immune system, particularly early developing B-cells, is still unclear. In this regard, it was of interest to evaluate and identify the role of elevated corticosterone accompanying ZD on the distribution of developing BM B-cells. Use of adrenalectomized ZD mice in which CS is eliminated clarified the role of this hormone in distribution of BM B-lineage cells. This study will be presented in Chapter Four.

Glucocorticoid-Induced Apoptosis:

The association of elevated levels of endogenous GCs with thymic atrophy and the subsequent suppression of immature thymocytes have been extensively documented in both humans and animals (Weissman, 1973; DePasquale-Jardieu and Fraker, 1979; Becker, 1983; Barone *et al.*, 1993). The cells affected in this system are predominantly the immature double-positive ($CD4^+$ $CD8^+$) thymocytes. Early studies considered this type of cell loss in thymocytes as cytolysis (Claman *et al.*, 1971). However, it is now clear that exposure of cells

to GCs initiates a range of events that leads the cells to "commit suicide." This type of cell death is termed programmed cell death (PCD) or apoptosis (Wyllie, 1980; Cohen, 1992).

Apoptosis (A_p) or PCD is a process responsible for selective deletion of cells during morphogenesis, embryogenesis, tumor regression, and tissue involution in response to chemical or physical stimuli (Wyllie *et al.*, 1980; Wyllie, 1987; Walker *et al.*, 1988). This form of cell death was first applied to cells dying physiologically without swelling, necrosis or inflammation by Kerr *et al.*, (1972). Since then many investigators have evaluated the morphological and molecular events in apoptotic cells in a wide range of tissues. Compared to necrosis, in which cells swelling is followed by rupture of plasma and organelle membranes and an inflammatory response, apoptotic death is unique and different. Based on histological examinations and electron micrographs, apoptosis is distinct from necrosis in various morphologic features: reduction in cell volume; condensation and margination of nuclear chromatin; maintenance of organelle integrity; blebbing of the cell surface and packaging of cytoplasmic organelles into membrane-bound fragments called apoptotic bodies; and lack of an inflammatory response. These alterations are accompanied by endonucleosomal cleavage of chromatin DNA into 180-200 base pair multimers also known as DNA ladders (Wyllie, 1987; Kerr *et al.*, 1987; Cohen, 1992). The DNA ladders are extensively used as a primary and predominant marker to identify apoptotic death (Telford *et al.*, 1991; Cohen and Duke, 1992; Schwartzman and Cidlowski, 1993).

Apoptosis is triggered by diverse signals including abnormal expression of oncogenes such as bcl-2, and c-myc, or tumor suppressor genes such as p53, and intracellular elevation of Ca^{+} concentration which activates nuclear Ca^{+}/Mg^{+}-dependent endonuclease activity

(Bissonnette *et al.*, 1992; Caelles *et al.*, 1994; Hermeking and Eick, 1994; Nicotera and Rossi, 1994). Besides the morphological features of apoptotic cells, the formation of the DNA ladders was primarily used to identify apoptotic populations. This was primarily achieved by determination of total and fragmented DNA in whole cell lysates either colorimetrically or by electrophoretic separation of low-molecular-weight DNA fragments on agarose gels (Wyllie, 1980). However, these techniques have the disadvantages of not quantifying the apoptotic proportion of a population and substantial time consumption (2-3 days). The use of flow cytometry in conjunction with surface and DNA immunofluorescent labeling provides a rapid, reliable and quantitative method of identifying subpopulations of cells within heterogeneous tissues such as BM that are apoptotic based on forward light scattering (size), and fluorescent intensity. This method was recently developed in our laboratory by William Telford and Louis King (Telford *et al.*, 1991). Using cell cycle analysis, they demonstrated that cells undergoing apoptosis accumulated in the so called hypodiploid region to the left of G_0/G_1 phase of the cell cycle, also termed A_0. This region (A_0) was also correlated with cells with fragmented DNA (DNA ladder) on agarose gels and morphological characteristics of apoptotic cells identified by electron micrographs (Telford *et al.*, 1991). Furthermore, using apoptotic inhibitors (eg., zinc, GR antagonist) the apoptotic peak (A_0) was eliminated, all of which confirmed the detection of true apoptotic cells by this rapid and highly quantitative method. This technique was extensively used in quantitation of apoptotic cultured thymocytes presented in Chapter Seven. In addition to the use of DNA binding dye for detection of A_0, recently more sophisticated techniques have been introduced for detection of this population. Among them, *in situ* nick translation of nucleotide analogs

into the DNA strand breaks and TdT labling of DNA breaks at their 3'-OH terminal with dUTP have shown to be more sensitive and more efficient methods compared to those previously described (reviewed by Telford *et al.*, 1994).

Among many inducers of apoptosis, glucocorticoid-induced apoptosis in mouse thymocytes has become a classical model in understanding and characterization of biochemical events in this type of cell death (Cohen, 1992). Apoptosis of immature thymocytes can be induced not only by GCs but also by anti-CD3 antibodies (Smith *et al.*, 1989; Shi *et al.*, 1991). Furthermore, removal of autoreactive T-cells and cells with nonfunctional gene rearrangement during thymic T-cell development have been shown to occur via apoptosis (Murphy *et al.*, 1990; Kisielow, 1995). Thus it seems that the sensitivity of different subsets of T-lineage cells to GCs is based on the level of differentiation and maturation, since the more mature single positive cells are much less sensitive (Telford *et al.*, 1991). This was further proved by Telford *et al.* (1994). Their *in vitro* studies of glucocorticoid-induced apoptosis in thymocytes demonstrated greater sensitivity of cells exhibiting $CD4^+CD8^+$ phenotype with low expression of TCR. However, single positive T-cells with high expression of TCR showed resistance to apoptotic death. The stage-dependent sensitivity of thymocytes has been also demonstrated *in vivo* (Sun *et al.*, 1992). Intraperitoneal injection of rats with dexamethasone (1 mg/kg), caused 50% thymic atrophy. A loss in thymocytes occurred within 2-8 hours after exposure to Dex primarily in only one fraction of the two main fractions isolated by percoll gradients. This fraction was shown to be immature thymocytes. This loss was accompanied by the appearance of small dense cells with characteristics of apoptosis falling in the hypodiploid peak on flow cytometric analysis.

Furthermore, cells eliminated in this fraction were presumed to be immature thymocytes (Sun *et al.*, 1992). These results suggested that the widely used *in vitro* model of glucocorticoid-induced thymocyte apoptosis closely mimics the *in vivo* events.

Although studies on glucocorticoid-induced apoptosis in B-lineage cells are limited, evidence indicate that B-cells show similar sensitivity to GCs as T-cells. In fact the elimination of autoreactive B-cells, B-cells with non-functional gene rearrangement at the transition state from pre-B cells to immature B-cells, and positive selection of B-cells with high affinity surface Ig for foreign antigens have been suggested to occur via apoptosis (Liu *et al.*, 1989; Rolink *et al.*, 1991; Scott, 1995).

Voetberg and colleagues demonstrated that the delivery of prednisolone (PD) to mice at a rate of a few nanograms per milliliter of plasma significantly reduced the proportion of early B-cells ($B220^+IgM^-$) and immature B-cells ($B220^+IgM^+$). To ascertain whether apoptosis played a role in elimination of these developing B-cells, BM cells were cultured in low levels of PD (0.1 μM) for 16 hours. Approximately 40% of cells expressing B220 ($B220^+$) and IgM (IgM^+) were located in the sub G_o/G_1 or A_o region of the cell cycle representing the apoptotic population. Further use of RU38486 eliminated the cells in A_o region confirming the GC-induced apoptotic death of this population (Voetberg *et al.*, 1994). This study demonstrated that both *in vivo* and *in vitro* chronic exposure of BM B-cells to low levels of PD diminshed the population of early and immature B-cells via apoptosis.

A similar study by Garvy and co-workers demonstrated a large proportion of murine BM $B220^+$ and IgM^+ (45-65%) B-cells underwent apoptosis when exposed to physiological levels of GCs for 12 hours in-vitro. Apoptosis was inhibited by high levels of zinc (500 μM),

a known inhibitor of apoptosis, and RU38486 (Garvy *et al.*, 1993b). Furthermore, *in vivo* CS pellet implantation in mice mimicking plasma CS levels detected during chronic stress (30-100 ml/dl) also indicated apoptotic loss of BM $B220^+$ and IgM^+ B-cells (Garvy *et al.*, 1993a). However, the apoptotic proportion of B-cells from CS treated mice was considerably lower than *in vitro* exposure of cells to the same apoptotic cue. This study demonstrated the rapid clearance of apoptotic cells over the first 24 hours after pellet implantation, since depletion of $B220^+$ cells at 50% of the controls diminished to 4% in the A_o region. This is expected since the rapid clearance of apoptotic cells by macrophages *in vivo* has been well documented (Wyllie *et al.*, 1980; Cohen, 1991). In fact, The rapid clearance of apoptotic cells by macrophages substantially prevents the leakage of toxic cell contents and local tissue injury. The degradation of ingested apoptotic cells by macrophages is remarkably fast as this process has been reported as short as 10-20 minutes (Savill *et al.*, 1989a; 1989b) or even shorter (Evan *et al.*, 1992) after the occurance of histological changes of apoptosis. This significant phenomenon has been considered in Chapter Five of this dissertation.

In addition to normal developing B-cells, some transformed B-cells also exhibit glucocorticoid-induced apoptosis. *In vitro* exposure of neoplastic B-cell lineage (B-chronic lymphocytic leukemia, B-cell) to methylprednisolone caused influx of Ca^+ followed by DNA fragmentation which are the characteristics of apoptosis. Furthermore, addition of protein synthesis inhibitor (cycloheximide) and RU38486 eliminated apoptosis in this population (McConkey et al., 1991). This observation indicates the pharmacological importance of GCs in the suppression of leukemic cells in leukemia.

Knowing the sensitivity of developing B-cells to GCs in both *in vivo* and *in vitro* and their elimination via apoptosis upon exposure to GCs (Voetberg *et al.*, 1994; Garvy *et al.*, 1993a; 1993b), it was of interest to examine whether or not the same phenomenon would occur in zinc deficiency where chronic elevation of GCs is present. This study will be presented in Chapter Five.

Figure 1: Biosynthetic pathways of adrenal steroid hormones (Hadley, 1992).

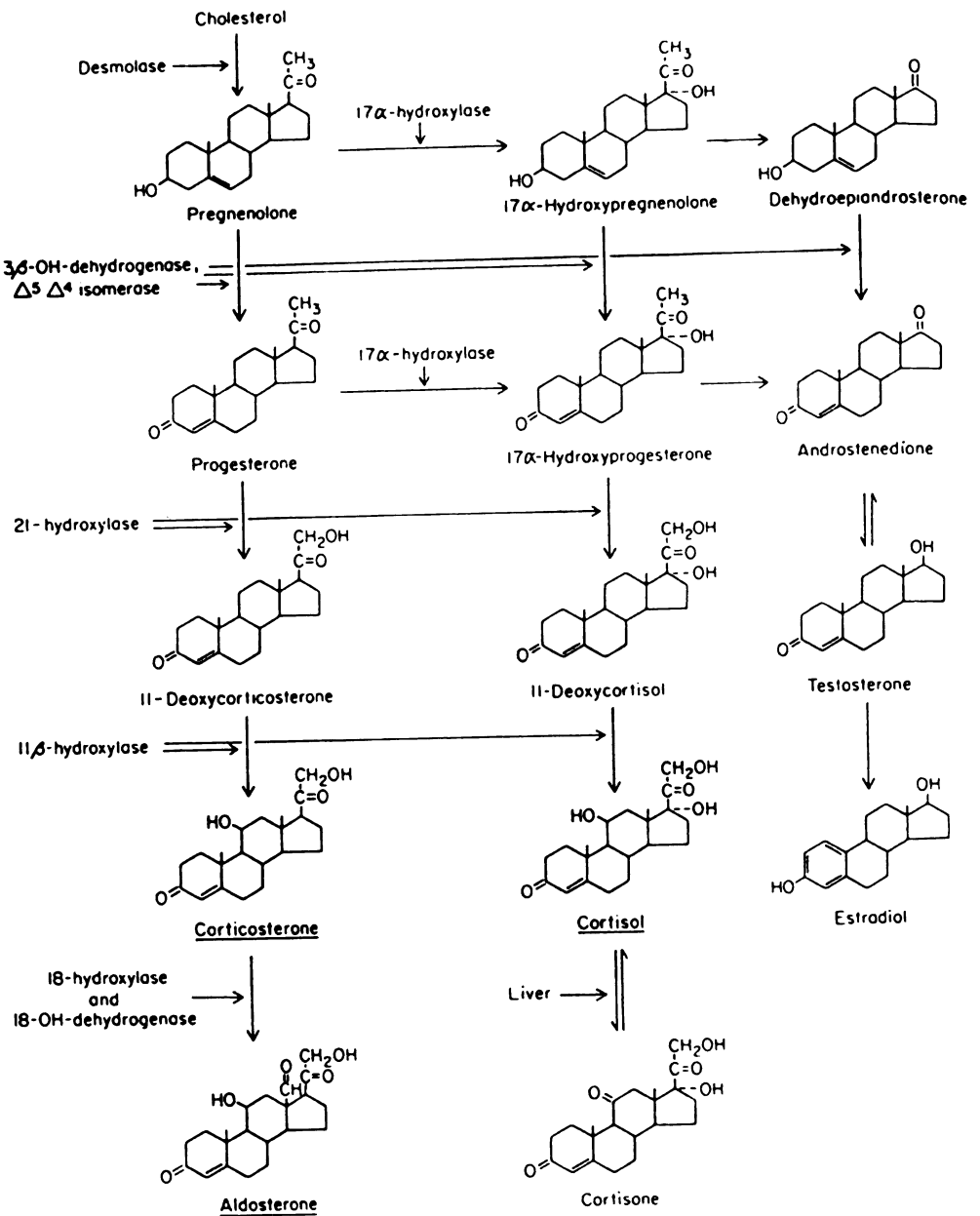

Cholesterol
Desmolase
Pregnenolone
17α-hydroxylase
17α-Hydroxypregnenolone
Dehydroepiandrosterone
3β-OH-dehydrogenase,
Δ5 Δ4 isomerase
Progesterone
17α-hydroxylase
17α-Hydroxyprogesterone
Androstenedione
21-hydroxylase
11-Deoxycorticosterone
11-Deoxycortisol
Testosterone
11β-hydroxylase
Corticosterone
Cortisol
Estradiol
18-hydroxylase
and
18-OH-dehydrogenase
Liver
Aldosterone
Cortisone

Figure 2: Hypothalamus-pituitary-adrenal axis; Regulation of glucocorticoid production.

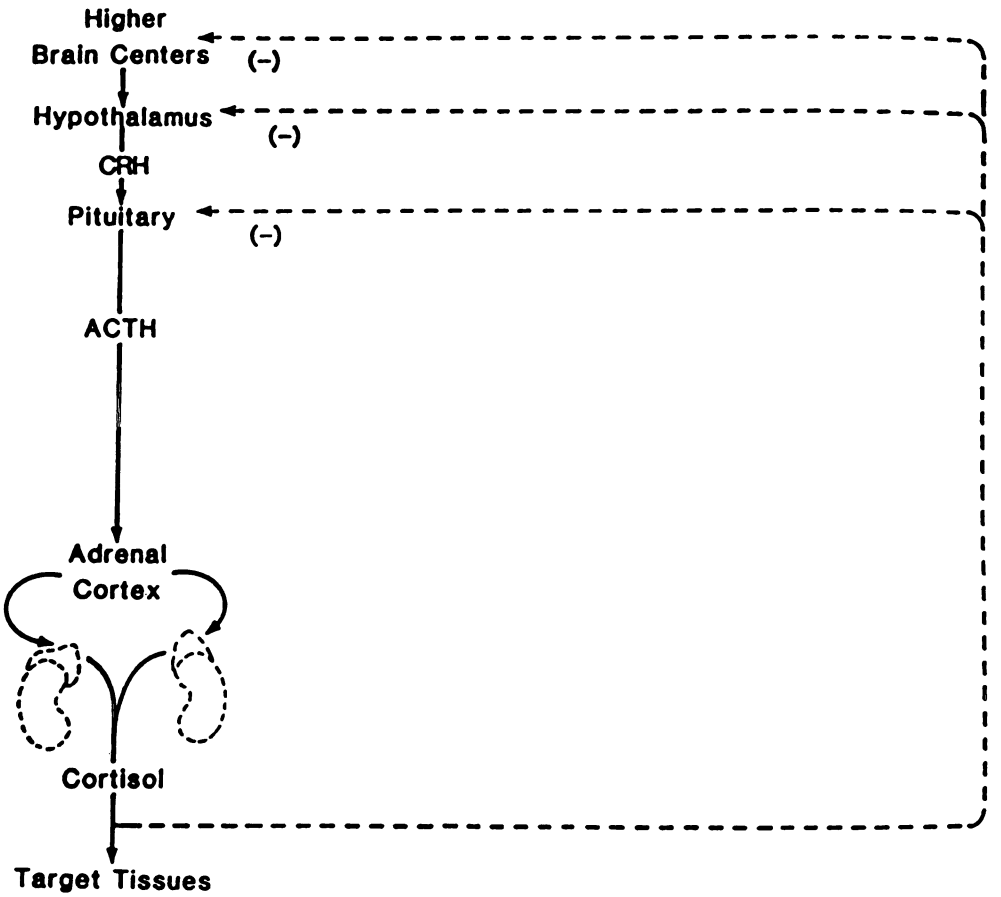

Higher
Brain Centers
(–)
Hypothalamus
(–)
CRH
Pituitary
(–)
ACTH
Adrenal
Cortex
Cortisol
Target Tissues

Figure 3: The functional domains of the steroid/nuclear (glucocorticoid) receptor.

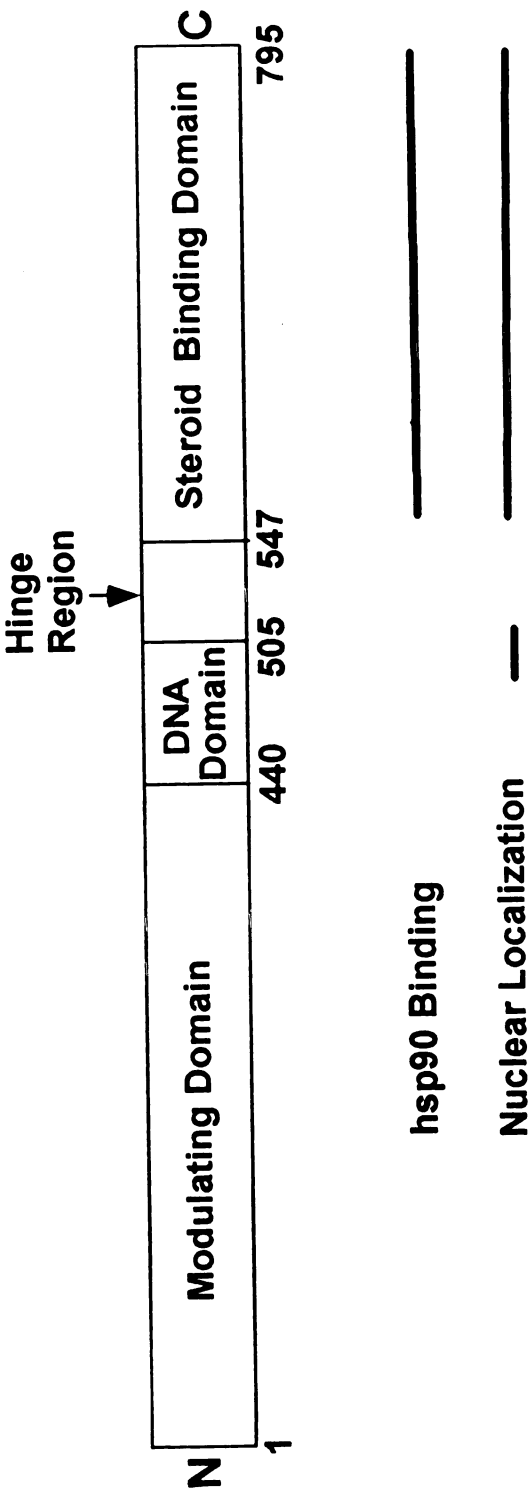
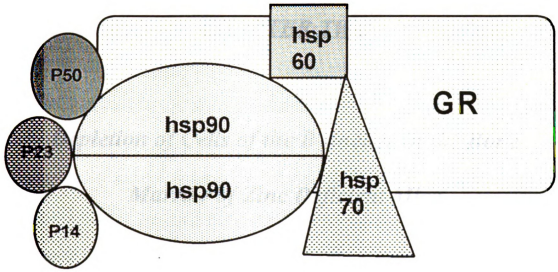

N
C
Modulating Domain
DNA Domain
Steroid Binding Domain
Hinge Region
1
440
505
547
795
hsp90 Binding
Nuclear Localization

Figure 4: Schematic representation of the glucocorticoid receptor heterocomplex in untransformed state.


hsp
60
P50
GR
hsp90
P23
hsp90
hsp
70
P14

CHAPTER TWO

Depletion of Cells of the B-lineage in the Bone Marrow of Zinc Deficient Mice

"A collaborative study with Dr. Louis E. King"

Abstract

Though lymphopenia is often noted in malnourished humans and rodents, little is known about the effects of suboptimal nutriture on lymphopoietic processes. Focusing primarily on cells of the B-lineage in the marrow of young adult mice, this study demonstrated that a moderate degree of zinc deficiency (MZD) caused a 43% decline in the proportion of nucleated cells bearing B220 with a 91% decline noted among more severely zinc deficient mice (SZD). Early B-cells ($B220^+Ig^-$) were highly sensitive to the deficiency, being barely detectable in SZD mice and reduced by almost 60% in MZD mice. Immature B-cells ($B220^+IgM^+IgD^-$) were similarly affected, declining 35% to 80% depending on the degree of the deficiency. In MZD mice, mature B-cells (IgM^+IgD^+) exhibited moderate losses, being somewhat resistant. A more profound loss in this population was noted for SZD mice. Flow cytometric (FACS) scatter profiles indicated that zinc deficiency caused a sharp decline in the proportion of small nucleated cells which in the marrow are thought to contain a high proportion of developing lymphoid cells. There was a concomitant increase in large granular cells which paralleled a substantial increase in the proportion of nucleated cells bearing Mac-1 for both MZD and SZD mice. Given the dramatic depletion of cells of the B-lineage in the marrow created by a deficiency in zinc, it is probable that disruptions in lymphopoietic processes in the marrow play a key role in the resulting lymphopenia observed in many types of malnutrition.

Introduction

Thymic atrophy and lymphopenia are often noted in malnourished humans and rodents (Endre *et al.*, 1990; Kuvibidila *et al.*, 1993; Fraker *et al.*, 1993; Cook-Mills and Fraker, 1993a). The resulting reduction in leukocytes, especially lymphocytes, suggests that some nutritional deficiencies might be altering bone marrow function and reducing its ability to produce lymphocytes. However, there is little information in the literature on this important topic. Therefore, the present study focuses on the effects of zinc deficiency, because it is a well characterized nutritional-immunological paradigm, on lymphopoietic processes in the marrow of young adult mice (Endre, *et al.*, 1990; Fraker *et al.*, 1993). Dietary deficiencies in zinc (ZD) are frequently noted in underdeveloped countries and occasionally in Western nations (Endre *et al.*, 1990; Walsh *et al.*, 1994). However, the deficiency also accompanies a variety of common disease states such as renal disease, alcoholism, chronic gastrointestinal disorders, sickle cell anemia, AIDS, certain cancers, etc. (Endre *et al.*, 1990; Keen and Gershwin, 1990; Walsh *et al.*, 1994; Prasad, 1995). Increased incidences of sepsis, respiratory infections, and various other secondary infections are seen in individuals with suboptimal intake of zinc, indicating the immune system is compromised (Endre, *et al.*, 1990; Keen and Gershwin, 1990; Fraker *et al.*, 1993; Hadden, 1995). The prevalence of this deficiency in the human population spawned interest in the development of rodent models for the study of zinc deficiency. In the case of the mouse, thirty days of an inadequate intake of zinc by young adult mice reduced thymic weights and splenocyte numbers 50% to 75% depending on whether the deficiency was moderate or severe (Cook-Mills and Fraker, 1993a).

Nevertheless, the phenotypic distribution or proportion of various subsets of mature T and B-cells were nearly normal even in the spleens of severely zinc deficient mice (King and Fraker, 1991). The residual mature splenic T and B-cells in the deficient mice responded appropriately to mitogenic and antigenic challenges producing normal levels of cytokines, antibodies, plaque forming cells, etc., when considered on a per cell basis (Cook-Mills and Fraker, 1993a). Indeed, reductions in the capacity of zinc deficient mice to respond to *in vivo* antigenic challenges directly correlated with reductions in the number of mature peripheral lymphocytes available to participate in such responses (Cook-Mills and Fraker, 1993a).

. Because the reduction in mature lymphocytes noted in zinc deficiency is substantial, and because zinc is known to play a key role in cell division and replication (Chesters, 1992; Vallee and Falchuk, 1993; Cousins, 1996), it seemed probable that the deficiency had altered lymphopoiesis in the bone marrow and thymus. The study herein represents the first detailed study of the effects of a nutritional deficiency on lymphopoietic processes, focusing on developing cells of the B-lineage. It shows that zinc deficiency causes significant losses in the proportion of cells of the B-lineage in the marrow that correlate with the degree of deficiency. This depletion of developing B-cells probably represents a seminal event in the eventual reduction of mature B-lymphocytes observed in the peripheral immune system as zinc deficiency advances. This is a collaborative work with Dr. Louis King who initiated this investigation and remained as a potential investigator throughout the study.

Materials and Methods

Mice, Diet, and Zinc Analysis:

Six week old A/J female mice weighing 17.1±0.1g (Jackson Labs, Bar Harbor, ME) were distributed into three dietary groups. The zinc adequate group (ZA) received diet containing 30 μg zinc/g diet, and the deficient group (ZD) received diet containing less than 0.7 μg zinc/g diet *ad libitum*. A third group, received zinc adequate diet restricted to the average quantity of food consumed the previous day by the ZD mice to control for inanition that accompanies the deficiency (RZA, restricted zinc adequate) (King and Fraker, 1991; Cook-Mills and Fraker, 1993a). All three dietary groups were housed in stainless steel cages with mesh bottoms to reduce recycling of zinc for a period of 28 days . Feed jars and bottles were washed in 4N HCl and the drinking water was acidified to reduce *Pseudomonas* infections. Mice in all three dietary groups were weighed weekly to establish the mean weight ± standard deviation. After a 28 day dietary period, ZD mice were subdivided into moderately affected mice by zinc deficiency (MZD), weighing 72%-76% of ZA mice and bearing a modest degree of parakeratosis of the eyes, ears, and tails; and severely affected mice by zinc deficiency (SZD), weighing 64%-68% of ZA mice and bearing extensive parakeratosis (King and Fraker, 1991; Cook-Mills and Fraker, 1993a). Zinc adequate and restricted zinc adequate mice selected for experimental analysis were randomly chosen to approximate the mean weight ± standard deviation for their respective group.

Blood Collection and Serum Zinc Analysis:

Blood collected from the subclavian arteries of anesthetized mice from all four dietary groups was processed individually in acid washed microtubes. For zinc analysis, serum samples were diluted 1:10 in 1% HCl and analyzed immediately by flame atomic absorption spectrophotometry (Varian AA-20 Plus, Mulgrave, Victoria, Australia) at 213.9 nm with deuterium background correction. A standard curve was established using concentrations of zinc ranging from 0.1 to 1 ppm (μg Zn/ml) prepared from an ultra-pure standard (Sigma, St. Louis, MO). Addition of known concentrations of Zn to serum samples (standard addition) resulted in greater than 90% recovery of zinc by this method.

Preparation of Mouse Bone Marrow Cell Suspensions:

Bone marrow from five to seven mice from each of the four dietary groups was flushed from femur and tibia and individually processed using harvest buffer (Hank's balanced salt solution containing 10 mM Hepes, pH 7.4 and 4% heat inactivated fetal bovine serum absorbed with mouse cells) (Garvy *et al.*, 1993a). Red blood cells were removed by centrifugation over a 3 ml Histopaq gradient (1.083 g/ml) (Sigma Chemical Co, St Louis, MO) except where noted. After washing, cells were resuspended in harvest buffer containing 0.15% sodium azide and kept at 4°C for phenotypic labeling and FACS analysis (Garvy *et al.*, 1993a). Cell viability at the completion of processing was greater than 95% by trypan blue dye exclusion.

Bone Marrow Phenotypic Labeling and Flow Cytometric Analysis:

Bone marrow from each mouse was processed separately and phenotyped in single and two color protocols as described below. Antibodies used were: B220 (RA3-6B2 isolated from ascites fluid by protein G column chromatography and biotinylated), IgM (μ chain specific and affinity purified, Tago, Burlingame, CA) or IgD (Fc specific, absorbed against μ chain, Nordic, CA). Single antibody phenotyping was used to identify changes in the proportion of all B-cells of the marrow ($B220^+$), immature-mature cells (IgM^+), and mature B-cells (IgD^+). Two color antibody combinations were used to identify specific B-cell subpopulation changes in the marrow such as early B-cells ($B220^+Ig^-$), immature B-cells (IgM^+IgD^-), mature B-cells (IgM^+IgD^+), etc. Antibody combinations were: 1) biotinyl-anti-B220/Strep-Avidin-phycoerythrin (AV-PE) vs anti-IgM/dichlorotriazinyl amino fluorescein (DTAF) for the detection of early B-cells versus immature-mature B-cells; 2) biotinyl-anti-IgD/Strep-AV-PE versus anti-IgM DTAF for the detection of immature and mature B-cells; 3) anti-B220-PE versus biotinyl-anti-Mac-1/Avidin-fluorescein-isothiocyanate (AV-FITC) for the detection of B-cells versus myeloid derived cells. One million marrow cells were labeled in the presence of harvest buffer plus 0.15% azide for 30 minutes at 4C with each reagent. The cells were analyzed immediately by flow cytometry.

One and two color flow cytometric analyses were done using an Ortho 50H Cytofluorograph/80386 computer system using Acqcyte software. Cells were selected for analysis based on presence in a scatter cytogram region consisting of low angle forward light scatter (channels 12-90, y-axis) vs orthogonal light scatter (channel 2-98, x-axis) which excluded cell debris and cell aggregates. Cells included in the scatter gate were examined for

the presence of antibody in single phenotype experiments or simultaneously examined for green (DTAF) and orange (PE) fluorescence in two color phenotypic experiments. Thymocytes were labelled in parallel, serving as negative controls. Background fluorescence found within positive fluorescence gates was subtracted from all data. In two color phenotyping less than 10% electronic color compensation was required to correct for spectral overlap in the detection of fluorochromes. FITC, DTAF, and PE were excited using the 488 nm line of an argon laser. FITC and DTAF emission was detected at 525 $\pm$ 10 nm and PE emission was detected at 570 $\pm$ 5 nm using band pass interference filters. The proportion of small nucleated cells in the marrow was determined by light scatter based on inclusion of greater than 80% of a thymocyte population within the small nucleated cell scatter cytogram region.

Statistical Analysis:

Nonparametric statistical analysis was used for all data presented. The nonparametric Kruskal-Wallis test was used to determine whether significant differences existed between treatment groups. Tukey's test, which does not presume normal data distribution, was used in posthoc analysis to determine which experimental groups were significantly different (Steel and Torrie, 1980). The mean $\pm$ standard deviation is presented in all cases.

Results

Effects of Zinc Deficiency on Body and Thymic weights and Serum Zinc Levels:

The resulting body weights for a typical 28 day dietary study are shown in Figure 1A where the average ZD and RZA mouse weighed 76% and 83%, respectively of ZA controls. The ZD mice were then subdivided into MZD and SZD based on body weights (~75% and ~65% respectively) and external signs of zinc deficiency as described in the Methods. Accordingly, thymic weights were nearly normal for RZA mice but exhibited extensive weight loss of 47% and 72%, respectively for MZD and SZD mice (Figure 1B), all of which is analogous to past dietary experiments. The zinc content of the serum was reduced 56% for ZD mice and were nearly normal among RZA mice (Figure 1C). As observed here, the zinc content of fluids and tissues often can not distinguish between degrees of deficiency though immune parameters often do (Endre *et al.*, 1990; Fraker *et al.*, 1993; Cook-Mills and Fraker, 1993a). MZD and SZD mice also exhibited more than a two-fold elevation in serum corticosterone levels compared to ZA mice (data not shown), which is also characteristic for zinc deficiency (DePasquale-Jardieu and Fraker, 1980; Fraker *et al.*, 1993).

Evaluation of Nucleated BM Cells and Their Phenotypic Distribution in the Marrow of Zinc Dietary Mice:

With regard to the bone marrow, scatter analysis of the nucleated cells by FACS showed statistically significant drops of 24% and 57%, respectively, for MZD and SZD mice in the proportion of small nucleated cells thought to contain large numbers of developing

lymphoid cells (Figure 2A) (Osmond, 1986). A sample scatter profile of the nucleated cells from the marrow of a ZA and a SZD mouse is provided (Figure 3). It clearly shows a marked depression in small nucleated cells with a concomitant increase in granular cells in SZD mice. A brief exploratory experiment indicated that cells of the myeloid lineage bearing Mac-1 ($B220^-MAC\text{-}1^+$) increased substantially. However, it correlated with the loss of B-lineage cells in MZD and SZD mice (Figure 2B). This fits with the scatter profiles (Figure 3) where an increase in the proportion of more granular cells (side scatter) was clearly observed in the marrow of SZD mice. Thus, the myeloid compartment is probably more resistant to the effects of zinc deficiency than differentiating marrow B-cells.

From Figure 4, it is evident that zinc deficiency had a profound effect on cells of the B-lineage. The proportion of cells of the B-lineage among nucleated cells of the marrow (total $B220^+$) declined 43% in MZD and 91% in SZD mice. Using dual color analysis, the following stages of B-cell development were also analyzed: (1) $B220^+Ig^-$, early B-cells containing pro and pre-B cells; (2) $B220^+IgM^+IgD^-$, immature B-cells; (3) IgM^+IgD^+, mature B-cells (Osmond, 1986; Hardy *et al.*, 1991). The early B-cell ($B220^+Ig^-$) population was most severely affected by ZD. MZD mice exhibited a 56% reduction in the proportion of this population, whereas the early B-cells were almost completely eliminated by SZD (4% of controls). The profound effect that nutritional deficiencies may have on early B-cells is further evident in the RZA mice, which exhibited a 40% drop in the proportion of this population even though their reduction in caloric intake was modest. It serves to demonstrate that reduced food intake that accompanies deficiencies in zinc in humans or rodents, also

contributes to changes in the immune system as previously shown (Prasad, 1991; Fraker *et al.*, 1993; Cook-Mills and Fraker, 1993a).

Immature B-cells ($B220^+IgM^+IgD^-$) were somewhat more resistant with no statistically significant decline in this population noted among RZD mice. Nevertheless, the immature B-cells declined about 35% in MZD mice. The severe form of the deficiency took a greater toll with a loss of 80% of immature cells among SZD mice (Figure 4). Mature B-cells normally account for only 3-5% of the marrow as is the finding here and thus, can be more difficult to measure quantitatively (Osmond, 1986). A small decline in this population of cells among MZD mice was repeatedly observed, though it was never statistically different from ZA mice (Figure 4). However, SZD mice experienced more than a 70% drop in the proportion of IgM^+IgD^+ bearing cells.

Discussion

The data presented herein indicate that 28 days of a suboptimal intake of a single essential nutrient, zinc, has a profound effect on lymphopoietic processes of the marrow. Both MZD and SZD caused significant depletions in the proportion of small nucleated cells of the marrow and of cells bearing $B220^+$ in particular. Early B-cells ($B220^+Ig^-$) were especially sensitive to suboptimal zinc with immature B-cells (IgM^+IgD^-) following closely in sensitivity. The more mature IgM^+IgD^+ cells, while harder to measure accurately, appeared to be somewhat more resistant to moderate levels of zinc deficiency. Thus, some resistance to the deficiency seemed to be acquired with lineage maturity. Experiments presented in Chapter Three will extensively examine the effects of the deficiency on progenitor (pro-B) and precursor (pre-B) B-cells in the marrow of mice in dietary zinc study. Whether or not the multipotent stem cells are affected by the deficiency is also of interest, though it is known at least some stem cells and/or early progenitor cells must survive since the immune system is almost completely rejuvenated within a two weeks of nutritional repletion in the case of MZD mice (Fraker *et al.*, 1978).

It seems clear that the ability of the marrow to produce and/or maintain lymphoid cells was severely compromised by ZD with gradations in magnitude of effects noted between the MZD and SZD mice. The rapid atrophy of thymus and reduction in thymic hormone activity previously observed during ZD would probably greatly hamper, if not prevent, the maturation of any early precursor T-cells that were generated in the marrow of the deficient mouse (Dardenne *et al.*, 1982). Thus, it is highly probable that production and/or maturation of both

B and T-cells are altered by ZD. This further suggests that changes in bone marrow function must at least partly account for the lymphopenia commonly observed in zinc deficient rodents and people (Fraker *et al.*, 1993; Prasad, 1995).

Since lymphopenia and thymic atrophy are frequently noted in both deficiencies of zinc and protein-calories (Endre *et al.*, 1990; Prasad, 1991; Kuvibidila *et al.*, 1993; Fraker *et al.*, 1993; Cook-Mills and Fraker, 1993a), one wonders if a purposeful down-sizing of this part of the immune system is set in motion as the nutritional deficiencies advance. The large number of lymphocytes that must be produced each day by the marrow would require high amounts of nutrients. As the body shifts from a well fed to a starved state, nutrients must be saved for truly vital tissues such as the brain, heart, liver, kidney, etc., so perhaps production of new lymphocytes must be reduced. In this light, it is interesting to point out that blood concentrations of the glucocorticoids cease their normal circadian rhythm and become chronically elevated albeit at modest levels during the course of zinc and protein calorie deficiencies in both humans and rodents (DePasquale-Jardieu and Fraker, 1980; Smith *et al.*, 1981; Fraker *et al.*, 1993; Kuvibidila *et al.*, 1993). Recently it was demonstrated that moderately elevated levels of corticosterone delivered over a ten day period analogous to the concentrations observed during ZD, caused substantial thymic atrophy (by day 3), and depleted the marrow of cells of the B-lineage in mice (by day 5) (Garvy *et al.*, 1993a). Early and immature B-cells were preferentially affected with some resistance noted among IgM^+IgD^+, as was observed for ZD mice (Garvy *et al.*, 1993a). Furthermore, such levels of steroid increased three to four fold the proportion of $B220^+$ cells in the marrow undergoing apoptosis (Garvy *et al.*, 1993a). *In vitro* 10^{-7}M cortisol induced 30% of $B220^+$ marrow cells

to undergo apoptosis in 8 hrs, further demonstrating that early B-cells are highly sensitive to glucocorticoids, and very prone to undergo steroid induced apoptosis (Garvy *et al.*, 1993b). In order to remove glucocorticoids from the equation, mice were adrenalectomized prior to placing them on ZA or ZD diets. It was found that in the adrenalectomized mice thymocytes were provided substantial protection during the course of zinc deficiency (DePasquale-Jardieu and Fraker, 1980). This finding prompted the question of whether adrenalectomy also provides protection for the cells of the B-lineage in ZD. This will be addressed in Chapter Four.

Though apoptosis appears to be one likely cause of depletion of B-cells during zinc deficiency, at least two other possibilities must also be considered: 1) reduction in the rate of B-cell production, and 2) disruption of the cell cycle of precursor B-cells. In first case, zinc deficiency could alter the activities of one or more zinc dependent enzymes (Vallee and Falchuk, 1993; Cousins, 1996) such that cell cycle progression would be lengthened. In the second case, zinc deficiency in combination with the initial rise in serum corticosterone could block the cell cycle. In support of the latter, this lab has previously shown corticosterone treatment significantly reduces the percentage of $B220^+$ B-cells in the S phase of the cycle (Garvy *et al.*, 1993a). In addition, the effects of zinc deficiency and corticosterone on B-cell cycle status and rate of lymphopoiesis are other important parts of the puzzle, and questions for upcoming projects in this laboratory.

Finally, the increase in granular cells noted in the enclosed scatter profiles of ZD mice, along with the greater proportion of $B220^-$ $Mac\text{-}1^+$ cells, fits with past observations that cells of the myeloid lineage are more prevalent in marrow exposed to glucocorticoid than are

lymphoid cells (Dexter *et al.*, 1977; Dexter and Testa, 1980). Since cells of the myeloid series provide substantial immune protection, being part of our first line of defense, their preservation may represent a sort of immunological "fail-safe" mechanism. Collectively, the data indicate a substantial sensitivity of early developing B-cells to the effects of ZD. Furthermore, they suggest that induction of the hypothalamus-pituitary-adrenocortical axis during zinc deficiency may play an important immunoregulating role by reducing production of new lymphocytes which require substantial amounts of nutrients.

Figure 1: The effect of zinc deficiency on body weights, thymus weights, and zinc content of serum. ZA, zinc adequate fed mice (control mice). RZA, restricted fed zinc adequate mice. MZD, moderately affected mice by zinc deficiency. SZD, severely affected mice by zinc deficiency mice. **(A)** Body weights for ZA, RZA, and ZD dietary groups during the 28 day dietary regime; n=8-24 per dietary population. Filled symbols identify experimental group weights for ZA, RZA, MZD, and SZD mice selected for further study on day 28. **(B)** Day 28 thymus weights by treatment group; n=5-6. **(C)** Serum zinc content of each treatment group at day 28; n=4-6. In all cases the group mean ± SD is shown. * $P \leq 0.05$ compared with the ZA group. Data are representative of three or more experiments.

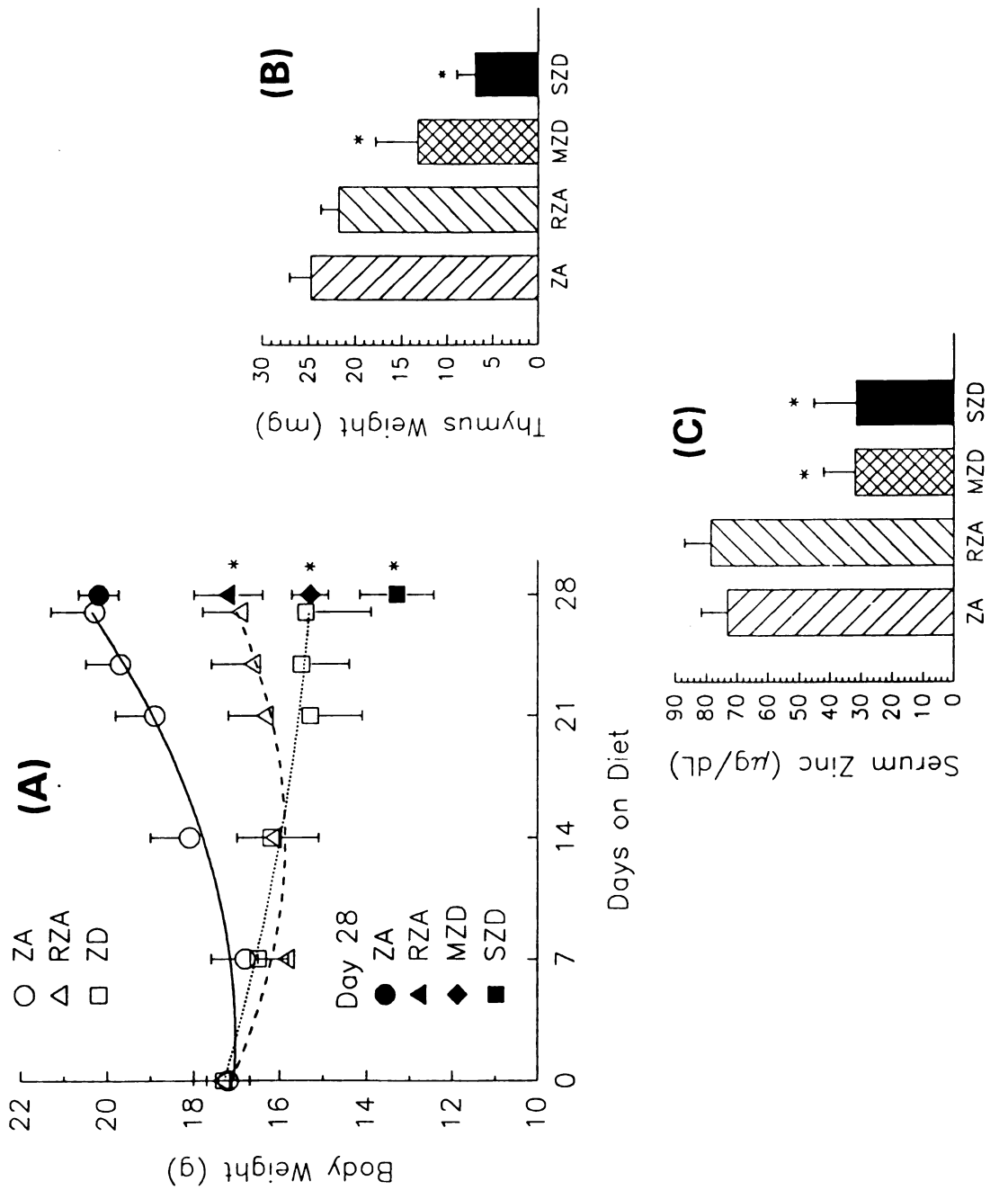

(A)
Body Weight (g)
Days on Diet
ZA
RZA
ZD
Day 28
ZA
RZA
MZD
SZD
(B)
Thymus Weight (mg)
ZA
RZA
MZD
SZD
(C)
Serum Zinc (μg/dL)
ZA
RZA
MZD
SZD

Figure 2: Assessment of effects of zinc deficiency on nucleated cells of the bone marrow by flow cytometry of mice from ZA, RZA, MZD and SZD groups after a 28 day dietary period. **(A)** Evaluation of size distribution of nucleated bone marrow cells using scatter analysis where thymocytes served as standard for small nucleated cells; n=5-6 per treatment group. Similar data were obtained from six previous experiments. **(B)** Phenotypic distribution of bone marrow $B220^+$ B-cells and $Mac\text{-}1^+$ myeloid cells among nucleated cells of the bone marrow; n=5-6 mice per group. * $P \leq 0.05$ compared with the ZA group where mean $\pm$ SD is shown.


(A)

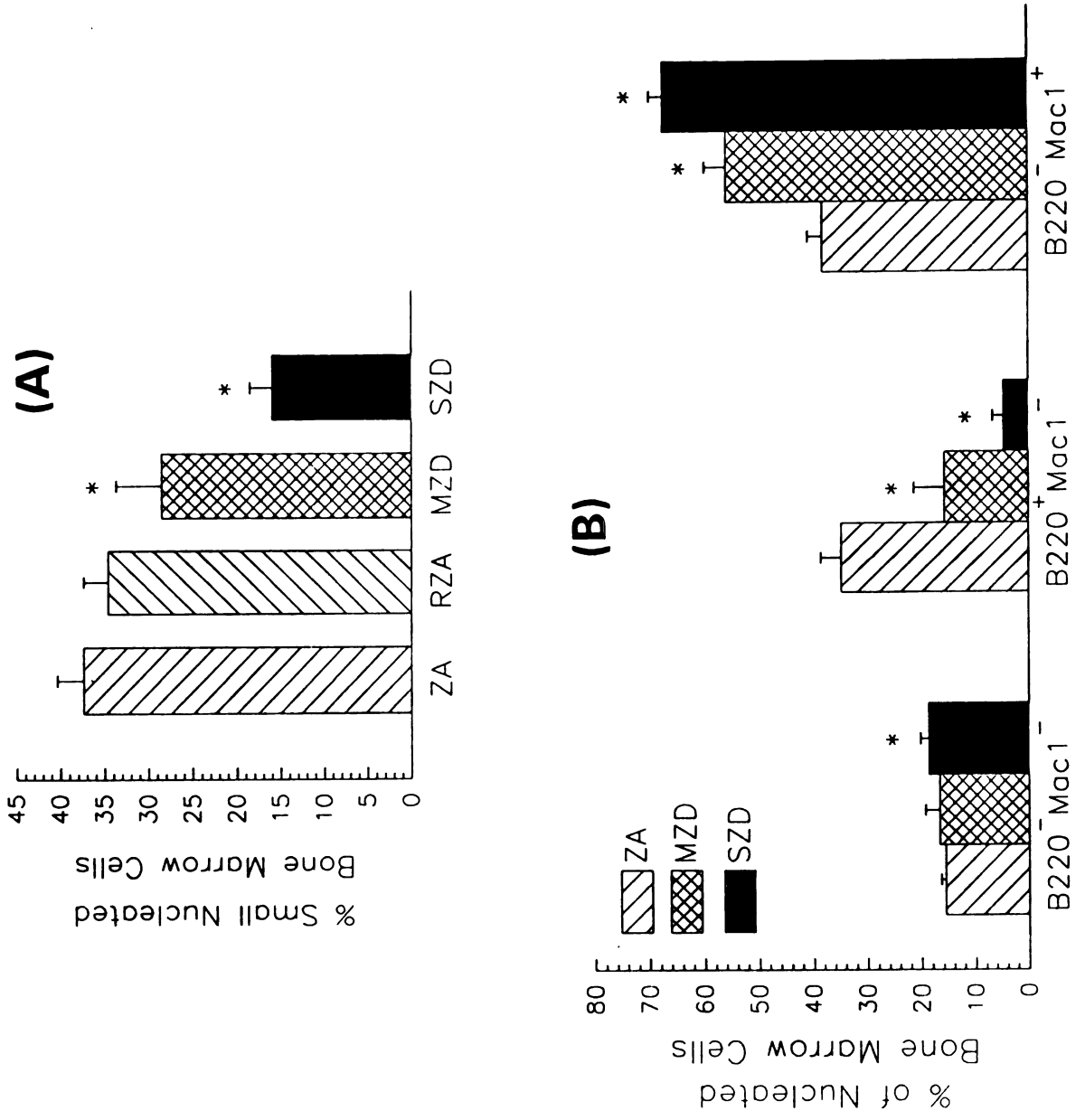
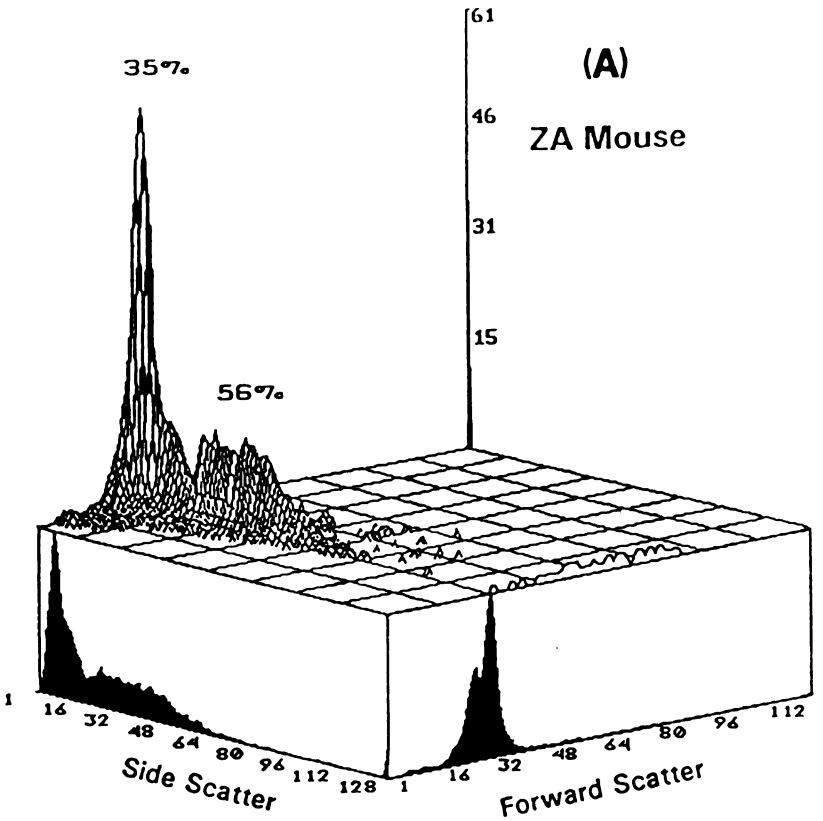

(A)
% Small Nucleated
Bone Marrow Cells
45
40
35
30
25
20
15
10
5
0
ZA
RZA
MZD
SZD
*
*
*
(B)
% of Nucleated
Bone Marrow Cells
80
70
60
50
40
30
20
10
0
ZA
MZD
SZD
B220⁻Mac1⁻
B220⁺Mac1⁻
B220⁻Mac1⁺
*
*
*
*
*

Figure 3: Flow cytometric light scatter profiles of nucleated cells of bone marrow prepared from normal and severely zinc deficient mouse after a 28 day dietary period. **(A)** ZA mouse. **(B)** SZD mouse weighing 68.5% of ZA controls and exhibiting extensive parakeratosis. The peaks at low forward and side scatter identify the heterogeneous small nucleated cell population of the bone marrow. The peaks at intermediate forward and high side scatter identify cells of the granular myeloid series in bone marrow.


(A)
ZA Mouse
35%
56%
61
46
31
15
1
16
32
48
64
80
96
112
128
Side Scatter
Forward Scatter

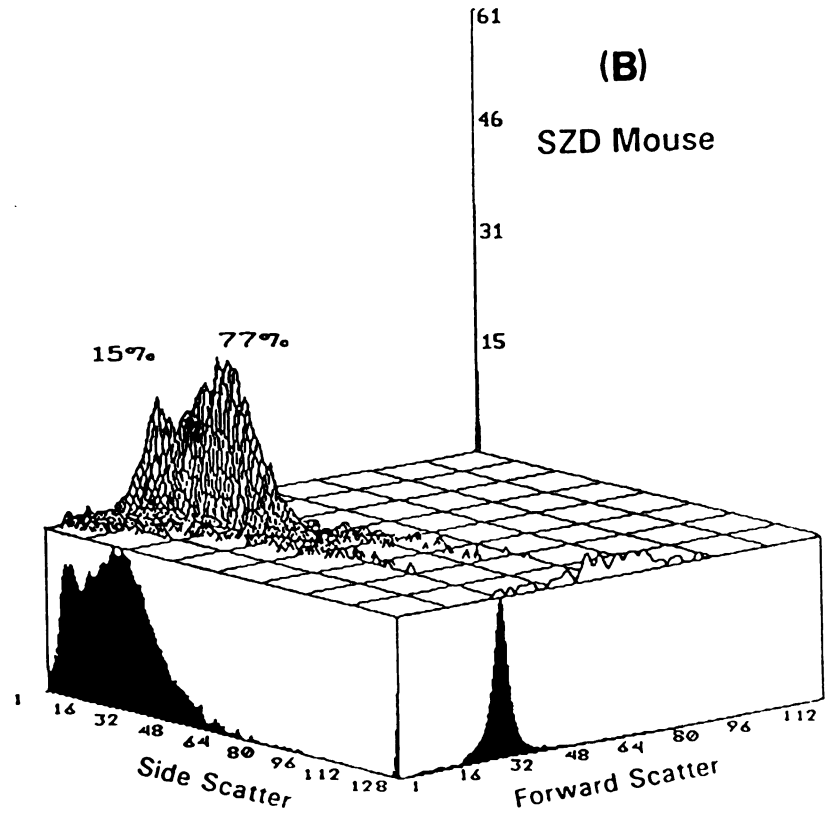
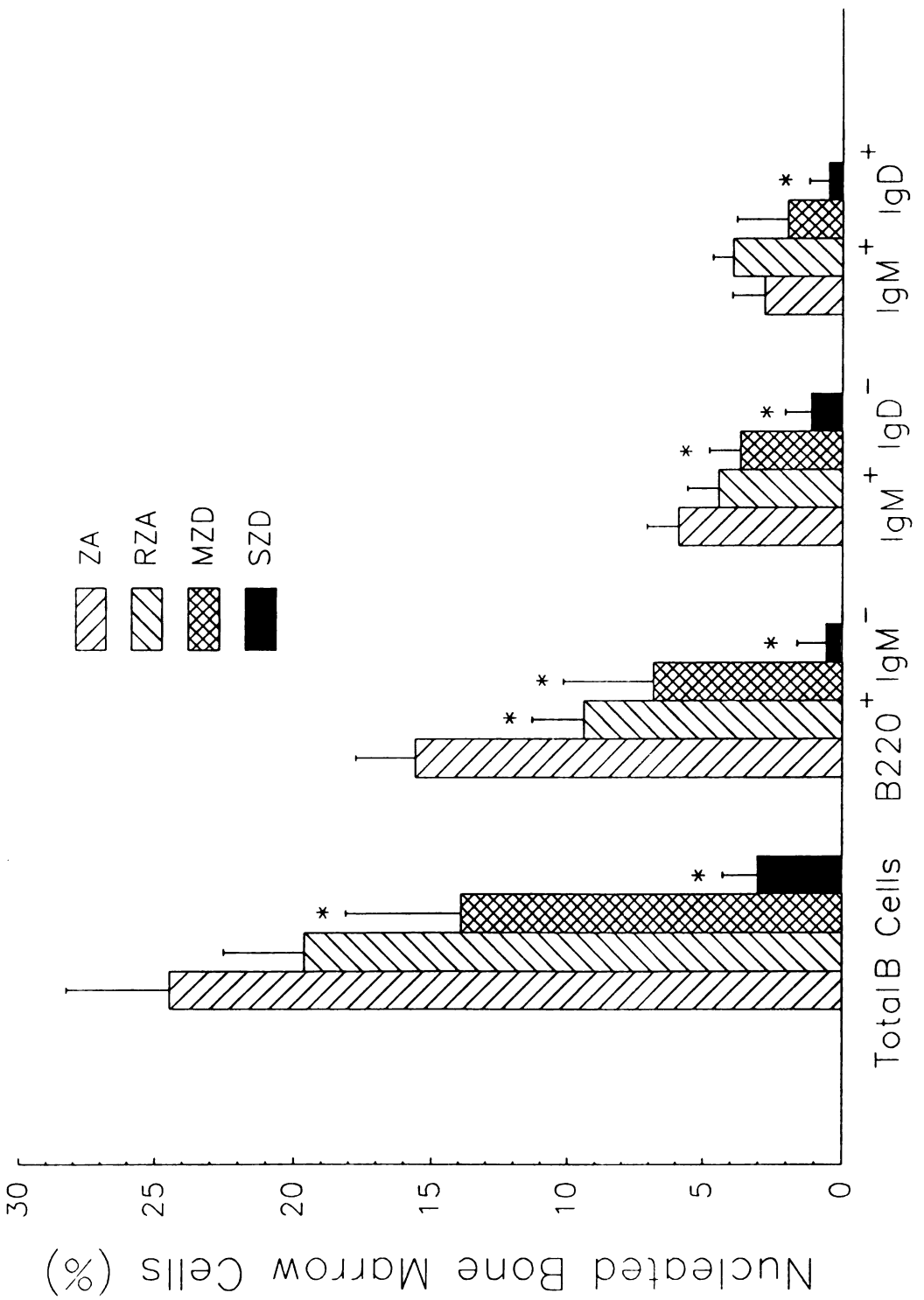

(B)
SZD Mouse
15%
77%
61
46
31
15
1
16
32
48
64
80
96
112
128
Side Scatter
Forward Scatter

Figure 4: Phenotypic distribution of early, immature, and mature cells of the B-lineage in marrow of ZA, RZA, MZD and SZD mice at day 28 by flow cytometry. The total B-cell population consists of all B-cells bearing B220 regardless of the degree of B-cell development ($B220^+$). The $B220^+IgM^-$ population defines the early B-cell population (pro and pre-B cells). The IgM^+IgD^- population defines the immature B-cell population. The mature B-cell population is composed of IgM^+IgD^+ B-cells; n=5-6 per group. $^*P \leq 0.05$ compared with the ZA group where mean $\pm$ SD is given. Data are representative of two experiments using two color analysis. The results are also supported by six single color experiments using anti-B220, anti-IgM, and four single color experiments for anti-IgD.


Nucleated Bone Marrow Cells (%)
30
25
20
15
10
5
0
ZA
RZA
MZD
SZD
*
Total B Cells
B220+ IgM−
IgM+ IgD−
IgM+ IgD+

CHAPTER THREE

Effect of Deficiencies in Zinc on the Status of Progenitor and Precursor-B cells in the Marrow of Mice

Abstract

Short periods of zinc deficiency (ZD) have adverse effects on immune system, causing thymic atrophy and lymphopenia in both humans and rodents. The previous chapter demonstrated that early B-cells ($B220^+Ig^-$) exhibited high sensitivity to the effects of ZD, with 60%-90% depletion depending on the severity of deficiency. Conversely, mature B-cells ($B220^+IgM^+IgD^+$) showed moderate losses, being somewhat resistant to the suboptimal dietary zinc. Whether the high sensitivity of different stages of early B-cells to ZD was stage specific was not clear. To investigate the status of early developing B-cells in the marrow of ZD mice, BM cells were immunofluorescently labeled with monoclonal antibodies (mAbs) directed to the key B-cell maturation surface markers (B220, CD43/S7, 6C3, and IgM) followed by flow cytometric analysis. Three color immunofluorescence staining of BM B-lymphocytes indicated 25% to 50% decline in B220 bearing cells, 50% to 70% decline in pre-B cells ($B220^+S7^-IgM^-$) and 30 % to 60% drop in immature/mature B-cells ($B220^+S7^-IgM^+$) (data not shown) of moderately and severely affected mice by zinc deficiency (MZD,SZD), respectively. However, early progenitor B-cells (early pro-B) ($B220^+S7^+6C3^-$) and late pro-B cells ($B220^+S7^+6C3^+$) showed only moderate losses in some cases being otherwise resistant depending on the severity of ZD in individual mice. Total cellularity of BM was also examined in all dietary groups and there were no significant differences among groups as the results of zinc deficiency. Evaluation of the zinc content of serum as well as some tissues detected 55% depression in the zinc content of serum in both MZD and SZD, and 32% to 39% decline in zinc level in leg bones of the MZD and SZD, respectively, compared to the

ZA

cel

rec

the

lyt

mo

im

is

ZA mice. Interestingly, there was no detectable change in the zinc levels of marrow or liver cells of ZD mice. The pattern of tissue zinc distribution observed in this study suggest the redistribution or mobilization of zinc in the body to maintain the survival and the function of the remaining cells. Taken together, the results indicate that the sensitivity of developing B-lymphocytes to ZD is stage specific and may depend on the degree of ongoing cellular and molecular activities which are zinc dependent. Furthermore, the role of other immunoregulatory factors (eg., GCs), secondarily to ZD, in the alteration of B-lymphopoiesis is suggested.

Introduction

Lymphopenia and thymic atrophy are hallmarks of many forms of malnutrition including zinc deficiency (ZD) (Endre *et al.*, 1990; Fraker *et al.*, 1993; Kuvibidila *et al.*, 1993). In previous studies, a strong correlation was noted between the loss in host defense capacity and losses in the number of lymphocytes populating the secondary immune tissues in zinc deficient adult mice. This suggested that alterations in the production of lymphocytes in the marrow might be an underlying cause of the lymphopenia so commonly observed in malnourished animals and people (King and Fraker, 1991). It seemed likely that insufficient zinc would alter lymphopoiesis since this essential trace element is critical to cell division, maturation, and developmental processes, all of which are integral to the production of lymphocytes by the bone marrow (Chesters, 1992; Vallee and Falchuk, 1993; Cousins, 1996).

The previous study presented in Chapter 2 examined the effects of a 30 day period of suboptimal intake of dietary zinc on young adult mice focusing on B-cell development in the marrow, which is the prime site for production and maturation of B-cells in all higher animals (King *et al.*, 1995). Using flow cytometric analysis it was evident that ZD substantially reduced the B-cell compartment by 40% to 90% depending on the degree of zinc deficiency. Substantial losses of 56% to 95% were noted among the so-called early B-cells ($B220^+Ig^-$) with losses of 35%-80% noted for immature B-cells ($B220^+IgM^+IgD^-$). The mature B-cells (IgM^+IgD^+) showed greater resistance to ZD with more moderate losses observed in this subset during the course of the deficiency. Clearly, suboptimal intake of zinc substantially altered the B-cell compartment of the marrow.

The current study expands upon the earlier work by employing three color flow cytometric analysis to specifically examine the effects of ZD on early and late pro-B cells as well as pre-B cells. It was of significance to know how ZD affected pro-B cells, with their immunoglobulin (Ig) genes still in the germline configuration, versus pre-B cells, which are actively engaged in rearranging the Ig genes (Rolink and Melchers, 1991; Li *et al.*, 1993). The serum zinc content and the intracellular zinc concentration of marrow, several lymphoid, and non-lymphoid organs along with analysis of the effects of ZD on the overall cellularity of the marrow also were investigated.

Materials and Methods

Mice and Diet:

Six week old female A/J mice (Jackson Laboratory Bar Harbor, ME) weighing 17.1±0.7 gm were provided a biotin fortified egg white based diet using AIN-93G vitamins and minerals (Reeves *et al.*, 1993) to which was added 30 μg Zn/g diet (zinc adequate group; ZA) or ~ 0.5 μg Zn/g diet (zinc deficient group; ZD) (Table 1). A restricted-fed group which was a control (RZA) for monitoring inanition that accompanies ZD, were fed zinc adequate diet (30 μg Zn/g diet) being limited to the average amount of food consumed by the zinc deficient group the previous day (Cook-Mills and Fraker, 1993a). All three dietary groups were maintained in stainless steel cages with mesh bottoms to reduce recycling of zinc for a period of 27 days. Feed jars and water bottles were washed in 4 N HCl and the drinking water was acidified to reduce *Pseudomonas* infections. Food consumption was recorded daily and body weights were measured twice a week. At the end of the dietary study, the ZD group was subdivided into moderately affected mice by zinc deficiency (MZD) weighing 72%-76% of the ZA group with moderate signs of parakeratosis of the ears and tail or severely affected mice by zinc deficiency (SZD) weighing 66%-68% of the ZA group which exhibited extensive parakeratosis per previous studies (King and Fraker, 1991; King *et al.*, 1995). The specific contents of the diet is presented in Table 1.

Blood Collection and Serum Zinc Analysis:

Blood collected from the subclavian arteries of anesthetized mice from all four dietary

groups was processed individually in acid washed microtubes. For zinc analysis, serum samples were diluted 1:10 in 1% HCl and analyzed immediately by flame atomic absorption spectrophotometry (Varian AA-20 Plus, Mulgrave, Victoria, Australia) at 213.9 nm with deuterium background correction. A standard curve was established using concentrations of zinc ranging from 0.1 to 1 ppm (μg Zn/ml) prepared from an ultra-pure standard (Sigma, St. Louis, MO). Addition of known concentrations of Zn to serum samples (standard addition) resulted in greater than 90% recovery of zinc by this method.

Tissue and Cell Preparations for Zinc Analysis:

The following procedures were used to avoid zinc contamination from the environment. Throughout sample preparation acid washed disposable plastic pipets, tubes and containers and EDTA washed surgical tools were used to reduce zinc contamination. Isotonic saline (0.9% NaCl) prepared from trace metal grade sodium chloride (SigmaUltra, Zn <0.0005%, Sigma chemical co.,St.Louis,MO) dissolved in milli Q water was used for cell suspensions. Tissues, including bone marrow, bones (devoid of marrow) and liver from mice in all dietary groups were processed and assessed individually. Marrow was flushed from femurs and tibias of 5-8 mice from each dietary group and suspended in isotonic saline. After removal of red blood cells (RBC) from extruded marrow by lysis (Coligan *et al.*, 1991a), the nucleated marrow cells were washed twice, and counted using Turk's nuclear staining solution (Whitlock *et al.*, 1984). They were then aliquoted at $1x10^7$ cells in acid washed tubes to be centrifuged at 400 xg for 5 min. The upper left liver lobe from each mouse was removed, rinsed in isotonic saline,

blotted, weighed and placed in acid washed tubes. Bones devoid of marrow followed the same procedure. An inductively coupled plasma-atomic emission spectroscopy (ICP/AES), a polyscan 61E simultaneous/sequential instrument (Thermo Jarnell Ash Corp, Franklin, MS) interfaced to a U-5000 ultrasonic nebulizer (Cetac Technologies Inc, Omaha, NB) was used for tissue zinc analysis (Nixon *et al.*, 1986). Marrow cells ($1x10^7$/sample) and whole tissues (liver and bones) were digested in 250 μl and 500 μl of Baker analyzed Ultrex II ultrapure nitric acid (Zn < 20 pg/g) (J.T.Baker Inc, Phillipsburg, NJ), respectively, for 6 hrs in 60-65°C waterbath. The samples were then cooled to room temperature and brought to a final concentration of 5% nitric acid by addition of Ultrex II ultrapure water (Zn < 100 pg/g) (J.T.Baker Inc, Phillipsburg, NJ).

The accuracy of the analysis was verified by comparing the readings obtained with standards from National Institute of Standards and Technology (NIST) multielement mix A-1 and multielement mix-B diluted to 1 ppm with 5% nitric acid. Quality control was achieved by using NIST bovine liver standardized for the ICP/AES method (Rajaram *et al.*, 1995). The ICP/AES values obtained from standards were within 97% to 98% of the NIST established range.

Preparation of Bone Marrow Cell Suspensions:

Bone marrow (BM) samples from all mice were processed and assessed individually. Marrow was flushed from femurs and tibias of 6-9 mice from each dietary group and suspended by gentle aspiration into Hepes buffered modified Hank's balanced salt solution (HBSS) supplemented with 4% fetal bovine serum (FBS). Red blood cells

were removed by density gradient centrifugation (Histopaq 1083, Sigma Chemical, St. Louis, MO). The nucleated cells at the interface were washed twice, counted, and tested for viability by trypan blue exclusion (>90% viable) (Coligan *et al.*, 1991b). To evaluate the absolute number of BM nucleated cells, marrow cell suspensions were prepared by the same investigator to provide consistency. In this case cells were immediately counted using Turk's nuclear staining solution (Whitlock *et al.*, 1984) without the use of any separation methods. All marrow cell suspension were kept at 4°C throughout processing.

Antibodies and Immunofluorescence Labeling and Flow Cytometry:

R-phycoerythrin (R-PE) conjugated rat anti-mouse CD45R (B220); fluorescein isothiocyanate (FITC) conjugated rat anti-mouse leukosialin (CD43/S7); biotin-conjugated rat anti-mouse 6C3 (Ly-6C) as well as the isotype-matched controls (biotin, PE or FITC conjugated rat IgG_{2a},κ) were all obtained from Pharmingen (San Diego, CA). Biotin-conjugated affinity-purified $F(ab')_2$ goat anti-mouse IgM which was μ-chain specific was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) while streptavidin Red 670 (R-670) was obtained from the Gibco BRL (Grand Island, NY). All the antibodies were used at their saturating concentration subsequent to titration.

For labeling with antibodies $1x10^6$ nucleated BM cells were resuspended in label buffer containing Hepes buffered modified HBSS containing 0.1% NaN3 with 2% FBS which was supplemented with 5% rat serum and 5% goat serum to reduce nonspecific binding. Cells were incubated in this mixture at 4°C for 20 min. After washing cells were then incubated in 150 μl of label buffer containing either anti-B220-PE, anti-CD43-

FITC, anti-IgM-biotin or anti-B220-PE, anti-CD43-FITC, anti-6C3-biotin for 30 min at 4°C and washed twice. To detect the biotinylated antibodies, an additional ½ hr incubation at 4°C with 100 μl of R-670 was required. Samples were then washed and the cells were suspended in 1 ml of label buffer, kept at 4°C being immediately analyzed on a Becton Dickinson Vantage equipped with a Consort 32 HP computer system with LYSIS II software. Fluorochrome excitation was accomplished using an ILT model RCP-50 argon laser tuned to 488 nm for FITC, PE, and R-670. Fluorochrome emission for FITC, PE, and R-670 was detected at 530$\pm$15 nm, 575$\pm$13 nm, and 670$\pm$14 nm, respectively. Negative controls included unstained cells to define the negative population and detect autofluorescence. Cells exposed to R-670 alone or labeled with isotype-matched non-specific antibodies were used to detect background fluorescence. Single and dual color controls were used to define the negative as well as the positive population for each antibody and to set color compensation. To calculate the background correction for each gated phenotypic population in the BM, a three color negative control composed of isotype-matched non-specific antibodies was used for each corresponding primary antibody that was used. Multistep gating was used to limit phenotypic analysis to lymphocyte populations using the following gates: cytogram gated BM cells which excluded debris and doublets (gate 1); B220 histogram gated selected for $B220^+$ cells (gate 2); and the cytogram scatter gated selected for lymphocytes only (gate 3). Cells which met these criteria were used for the phenotypic analysis.

Statistical Analysis:

All data were analyzed by the Kruskal-Wallis nonparametric test and the analysis of variance (ANOVA) for parametric data in order to identify significant differences between dietary groups (Daniel, 1987). A Tukey's post-hoc test was then applied to locate differences among the means of the dietary groups (Daniel, 1987). Differences were considered statistically significant at $P < 0.05$. All data are presented as the mean $\pm$ SD where n is 6 to 9 mice unless otherwise indicated.

Results

Effects of ZD on Body Weight, and Thymus Weight:

Figure 1 illustrates the changes in body weight and thymus size for all dietary groups. By day 27, the average ZD mice weighed 70% of the ZA controls while RZA mice showed no statistically significant decrease in body weight (Figure 1A). Prior to the termination of diet study, mice in the ZD group were subdivided into MZD and SZD groups based on the degree of body weight (72%-76% and 66%-68% of the ZA group, respectively) (Figure 1A) and the severity of skin parakeratosis as described in Materials and Methods. With this subdivision, MZD mice exhibited an almost 50% decline in thymus weight, while SZD mice exhibited severe thymic atrophy or a 70% decline in weight (Figure 1B). RZA mice had nearly normal thymic weights (Figure 1B).

Effects of zinc deficiency on serum and tissue zinc distributions:

The zinc content of sera (2A), nucleated BM cells (2B), liver and bones devoid of marrow (2C) of mice from all dietary groups on a 27 day of the diet study are shown in Figure 2. The serum zinc in both MZD and SZD groups declined similarly to about 41% of that in the control ZA group, whereas RZA group showed no statistically significant change in their serum zinc content (Figure 2A). The lack of distinction in serum zinc levels between MZD and SZD groups has been noted in previous studies (King and Fraker, 1991; Cook-Mills and Fraker, 1993a; King *et al.*, 1995). By contrast, mice from all four dietary groups showed the same zinc concentration for nucleated cells from the

marrow (Figure 2B). Likewise, the liver zinc content showed no significant difference among mice from the four dietary groups (Figure 2C). However, the zinc level in the bone declined significantly in the ZD groups, showing a 32% decline in MZD, and a 39% in SZD compared to that of ZA group (Figure 2C).

Effects of ZD on Progenitor B-cells:

To evaluate the effects of ZD on early stages of B-cell development, Hardy's scheme of B-cell development in murine system ,illustrated in Figure 3, was extensively used. Multiple three color phenotyping of BM cells using monoclonal antibodies against B-cell differentiation antigens (eg., B220, S7, 6C3, IgM) were carried out and analyzed using a dual laser flow cytometer. In the first set of three color phenotyping, $B220^+$ gated BM B-lymphocytes were screened for the expression of CD43/S7 and/or IgM molecules. This approach enabled us to identify multiple stages of B-cell development to include: early B-cells ($B220^+IgM^-$), pro-B cells ($B220^+S7^+IgM^-$), pre-B cells ($B220^+S7^-IgM^-$), immature and mature B-cells ($B220^+S7^-IgM^+$) (Hardy *et al.*, 1991). As shown in Figure 4A, the proportion of all cells of the B-lineage ($B220^+$) in the marrow was reduced by approximately 25% in MZD mice and 50% in SZD mice. The pre-B compartment of the BM normally comprising 12-15% of the nucleated BM cells was affected the most by ZD declining almost 50% in MZD mice, and 70% in SZD mice. Interestingly, in contrast to the pre-B cells, the pro-B cell population comprising 4%-6% of the nucleated BM cells exhibited significant resistance to the deficiency remaining at near normal levels in the marrow of both MZD and SZD animals (Figure 4A). In agreement with these findings,

the flow cytometric scatter profile of total $B220^+$ cells from the marrow of a ZA and a SZD mouse is presented in Figure 5. It clearly demonstrates a significant depression in $B220^+$ gated lymphocyte population (right panels), as well as precursor B-cells with relative resistance of progenitor B-lymphocytes in the marrow of SZD mouse (left panels).

Since the pro-B cells could be further divided into subcompartments, another set of three color phenotyping was used, replacing the biotinylated-anti-IgM with biotinylated anti-6C3 (Hardy *et al.*, 1991) (Figure 4B). The 6C3 (BP1) molecule is a cell surface glycoprotein whose expression is limited to early B-lineage cells in hematopoietic tissues and is not found on mature B-lymphocytes. This new combination of antibodies subdivided pro-B cells into early pro-B cells ($B220^+S7^+6C3^-$) and late pro-B cells ($B220^+S7^+6C3^+$) as well as pre-B cells ($B220^+S7^-6C3^+$) and immature and mature B-cells ($B220^+S7^-6C3^-$). Pre-B cells, as expected, were significantly affected by ZD showing 50% to 70% decline in both MZD and SZD mice, respectively (Figure 4B). As demonstrated in Figure 4B, evaluation of total pro-B cells and late pro-B compartments indicated no significant decline in these populations in either the MZD and SZD mice. There was, however, a moderately significant loss of early pro-B cells in SZD mice, but not MZD mice. Clearly, pre-B cell precursors were much more affected by ZD than pro-B cells.

The phenotypic data presented above was expressed as a percentage of the total population of nucleated cells found in the BM. To better demonstrate overall degree of the change in the early B-cell compartment during ZD, the pro and pre-B cell populations of the BM were expressed as a proportion of the B-cell compartment itself. As can be seen

in Figure 6, the differences in sensitivity of pre-B cells and pro-B cells to ZD are highlighted using this type of analysis. The significant depletion of pre-B cells detected in MZD and SZD groups (26% and 47%, respectively) was concomitant with a moderate accumulation of pro-B cells for MZD (+10%) and SZD (+43%) groups. This more clearly shows the severity of depletion of pre-B cells by ZD and the much higher resistance of the pro-B cell population to ZD.

Effects of ZD on BM Cellularity:

Given the significant decline in the number of cells in the B-cell compartment, we were also interested in the effects of the deficiency on the overall cellularity of the BM. To investigate this parameter, marrow suspensions were prepared from mice in all dietary groups in order to determine the total number of nucleated BM cells per two legs. Interestingly, the data showed almost no change in the overall cellularity of the BM of mice in any of the dietary groups. Thus, in spite of depletion of the B-cell compartment in the marrow during ZD, the overall BM cellularity was not significantly altered (Figure 7).

Discussion

The results herein confirm results from the previous study (Chapter 2) in which depletion of total nucleated BM B-cells as well as early B-lineage cells in the course of 28 days of dietary zinc deprivation in mice were demonstrated (King *et al.*, 1995). It furthermore identifies the effects of ZD on the status of a small subpopulation (4-6%) of early B-lineage cells, namely, progenitor B-cells that are the earliest committed cells in the B-lineage and therefore the key to the B-cell development. The results of this study indicate a selective and stage-specific sensitivity of B-lymphopoiesis to the effects of zinc deficiency in the marrow of young adult mice. The BM cells bearing B220 surface antigen (total B-cells) demonstrated a 25% to 50% decline (based on the severity of deficiency) in zinc deficient mice. Utilizing Hardy's scheme of B-cell development (Hardy *et al.*, 1991) in subdividing the early B-lineage cells into subpopulations (Figure 3), a significant depletion of precursor B-cells (pre-B) ($B220^+S7^-IgM^-$) was noted in both MZD (50%) and SZD (70%) groups indicating their high sensitivity to the effects of suboptimal zinc intake. The progenitor B-cells (pro-B) ($B220^+S7^+IgM^-$), on the other hand, demonstrated a substantial resistance to the effects of ZD with the exception of the early pro-B cells ($B220^+S7^+6C3^-$) in the SZD group which showed a moderate but statistically significant loss. These data fit the scatter profiles (Figure 5) where a remarkable depletion in the total lymphocyte population due to significant alteration in the phenotypic distribution of pre-B and immature/mature-B cells and only a moderate decline in pro-B cells, in SZD mice were noted. Furthermore, the extensive loss of pre-B cells and the protection of pro-B

cells in the course of zinc deficiency were even more magnified when these populations were expressed as proportions of the B-cell compartment.

This unique stage specific effect of ZD on BM B-cell development could be evaluated from different prospective views. It is well documented that zinc is an essential element involved in DNA, RNA and protein synthesis, and gene expression. Zinc also is a structural or functional component of many metalloenzymes (Endre *et al.*, 1990; Vallee and Falchuk, 1993; Cousins, 1996). With this in mind, this study evaluated two early B-cell populations which exhibit different levels of biochemical activities. Progenitor B-cells, on one hand, are the most immature B-lineage committed cells which retain Ig genes in the germline configuration; they exhibit low turnover rate with an average of $2.5x10^6$ cells/day (Hardy *et al.*, 1991; Li *et al.*, 1993; Osmond *et al.*, 1994). Thus the survival of this population is a key to the B-cell development. Precursor B-cells, on the other hand, are actively cycling cells with an average turnover rate of $28x10^6$ cells/day (Osmond *et al.*, 1994). This pool is heavily involved in the Ig gene rearrangement, gene expression and protein synthesis, all of which are required for successive progression of cells from this stage to the next stage of B-cell development (Rolink and Melchers, 1991; Li *et al.*, 1993). Therefore, the bioavailability of zinc for these biological activities and survival of this population may be more crucial.

Furthermore, the notion of stage-specific expression of Bcl-2 oncogene in developing B-cells and its correlation with glucocorticoid-induced death (Merino *et al.*, 1994; Nunez *et al.*, 1994; Cory, 1995) could also play a role in the observed pattern of B-cell development in the course of zinc deficiency. Previous studies from our lab have

shown the modulation of the stress axis (hypothalamus-pituitary-adrenocortical axis) via zinc deficiency and the subsequent chronic elevation of glucocorticoids in the circulation in mice (DePasquale-Jardieu and Fraker, 1979; 1980). A 10-day *in vivo* delivery of corticosterone analogous to the concentrations detected during zinc deficiency caused substantial depletion of B-cells, preferentially early and immature B-cells (Garvy *et al.*, 1993a). In addition, this chronic elevation of corticosterone caused a three to four fold increase in the proportion of B-cells in the apoptotic region (Garvy *et al.*, 1993a). Moreover, the recent study by Merino *et al.* (1994) regulation of Bcl-2 in B-cell development, demonstrated high expression of the product of the Bcl-2 protooncogene, a known inhibitor of apoptosis, in progenitor B-cells and its downregulation in precursor and immature B-cells. They further showed the sensitivity of pre-B cells and the resistence of pro-B cells to dexamethasone induced apoptosis and their correlation with the Bcl-2 levels. Thus, chronic elevation of glucocorticoids during zinc deficiency along with changes in expression of Bcl-2 at different stages of B-cell development could be considered as another possibility for depletion in one case (pre-B) and protection in another (pro-B). In this regard, the significant depletion of pre-B cells and the substantial resistance of pro-B cells observed in ZD mice could be a reflection of their sensitivity to the elevated level of GCs induced by insufficient dietary zinc intake along with their level of Bcl-2 expression.

In the case of serum zinc determination, the data confirms the previous reports in which serum zinc depletion has been repeatedly observed in both humans and rodents suffering from ZD (Endre *et al.*,1990; Walsh *et al.*, 1994; King *et al.*, 1995). However, the same level of depletion in serum zinc concentrations in both MZD and SZD as

observed in this study is indicative of problems observed in both human and rodent research where plasma or serum zinc content give only a relative idea of zinc status without indicating the severity of the deficiency (Cook-Mills and Fraker, 1993a; Walsh *et al.*, 1994; King *et al.*, 1995). In regard to tissue zinc analysis, bone which is known as one of the largest mobilizable zinc pools (Jackson, 1989), showed significant drop (32% to 39%) in its zinc content in both MZD and SZD mice. This is possibly due to the redistribution of zinc from this tissue to other vital tissues such as the liver in the course of zinc deficiency (Brown *et al.*, 1978; Masters *et al.*, 1983; Guigliano and Millward, 1984), as there is no bodily store for zinc (Golden, 1989). Soft tissues such as marrow and liver of ZD mice, on the other hand, did not demonstrate any change in their zinc content and still maintained the same levels of zinc detected in ZA mice. This suggests that the survival and function of the cell machinery particularly in vital tissues, is based upon a continuuous inflow of nutrients. Thus, in situations where zinc becomes suboptimal, extracellular sources such as serum and mobilizable sources such as bone would be the available sources of zinc redistribution for vital tissues to survive (Jackson, 1989). Alternatively, the similar zinc content in soft tissues of all dietary groups could suggest the contribution of other factors in the disruption of lymphopoiesis; factors such as elevated levels of GC secondary to the ZD as well as overexpression or downregulation of some oncogenes (e.g. Bcl-2) which have been shown to regulate cell survival (Merino *et al.*, 1994; Cory, 1995; Fraker *et al.*, 1995).

Finally, the unchanged cellularity of BM in all dietary groups indicates the survival of other cell types such as myeloid lineage in the BM of zinc deficient mice, as was

demonstrated in Chapter 2 (King *et al.*, 1995). It has been shown that the *in vitro* survival of myeloid lineage (Dexter culture condition) is based upon GCs (hydrocortisone) supplementation to the culture system (Dexter and Testa, 1980). Thus, in ZD, where chronic elevation of GCs is reported (DePasquale-Jardieu and Fraker, 1979; 1980; Fraker *et al.*, 1995; King *et al.*, 1995), survival of cells of myeloid lineage (King *et al.*, 1995) is expected.

Taken together, these observations strongly indicate that the effects of ZD upon B-cell development is stage specific. The sensitivity of pre-B cells and the resistant of pro-B cells to the effects of ZD could be due to the degree of biological activity and related zinc requirements. Furthermore, the stage specific expression of molecules such as Bcl-2 in B-lymphocytes could regulate B-cell survival by protecting their elimination via GC-induced apoptosis which is thought to happen in ZD. Finally, the pattern of zinc distribution in different tissues of ZD mice suggest the redistribution of zinc in the body to maintain homeostasis and biochemical activities in vital tissues which are key to the survival.

Table 1: Composition of the Diet

Ingredients	g/kg diet	Percent diet	Source
Glucose monohydrate	609	60.9	Harlan/Teklad, Madison, WI
Egg white solids	200	20.0	Harlan/Teklad, Madison, WI
Corn oil	100	10.0	Michigan State Uni., Food Services
Salt mix[1]	40	4.0	Bioserv Inc., Frenchtown, NJ
Fiber[2]	30	3.0	Bioserv Inc., Frenchtown, NJ
Vitamin mix[3]	10	1.0	Bioserv Inc., Frenchtown, NJ
Biotin-premix[4]	10	1.0	Made in house
Ethoxyquin[5]	1	0.1	Monsanto Chemical Co. St.Louis, MO
Total	1000	100	

[1] AIN-93G mineral mix without zinc carbonate, supplemented with appropriate amount of zinc carbonate added in house to the zinc adequate diet to make it ~30 μgZn/g diet.

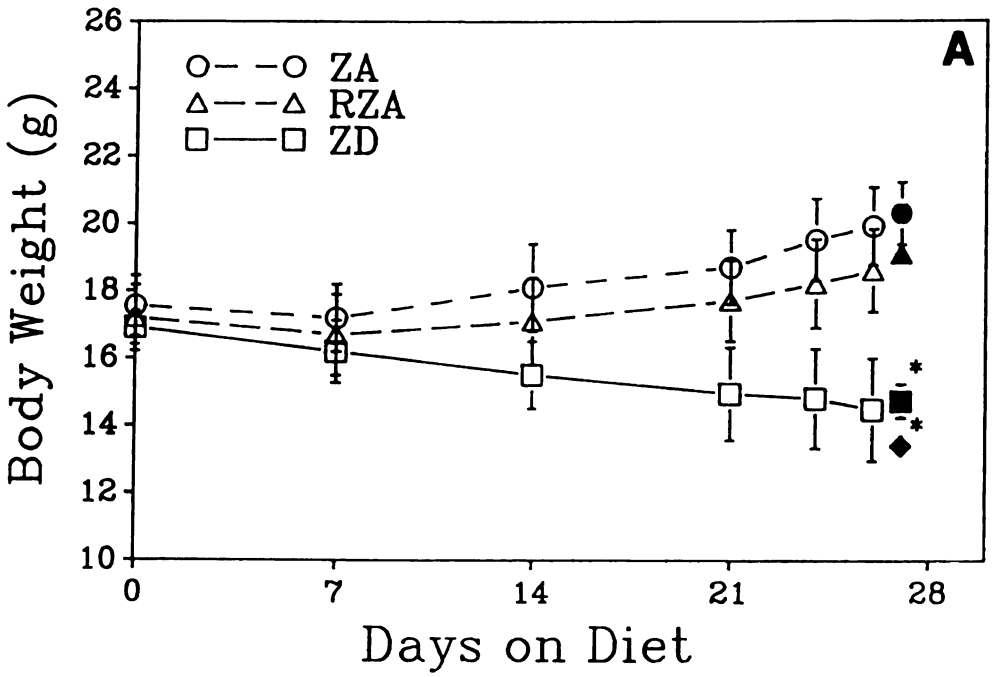
[2] Cellulose-type fiber.

[3] AIN-93 vitamin mix

[4] One part d-biotin mixed with five parts glucose monohydrate to offset the avidin found in the egg white protein.

[5] Ethoxyquin or santoquin for prevention of oxidation of polyunsaturated fatty acids.

Figure 1: Effect of ZD on body and thymus weights for the 27 day dietary study. **(A)** Changes in body weights of ZA (○---○), RZA (Δ---Δ) and ZD (□---□) dietary groups at various time points during the dietary study. Filled symbols represent final mean body weights of mice selected for the ZA (●), RZA (▲), MZD (■), and SZD (♦) groups at day 27. **(B)** Average thymus weights for each dietary group at day 27. ZA, n=7; RZA, n=5; MZD, n=9; SZD, n=8. Data are mean±SD being representative of 3 separate experiments. * Denotes data significantly different from ZA group at $p < 0.05$.


A
ZA
RZA
ZD
Body Weight (g)
26
24
22
20
18
16
14
12
10
0
7
14
21
28
Days on Diet
*
*

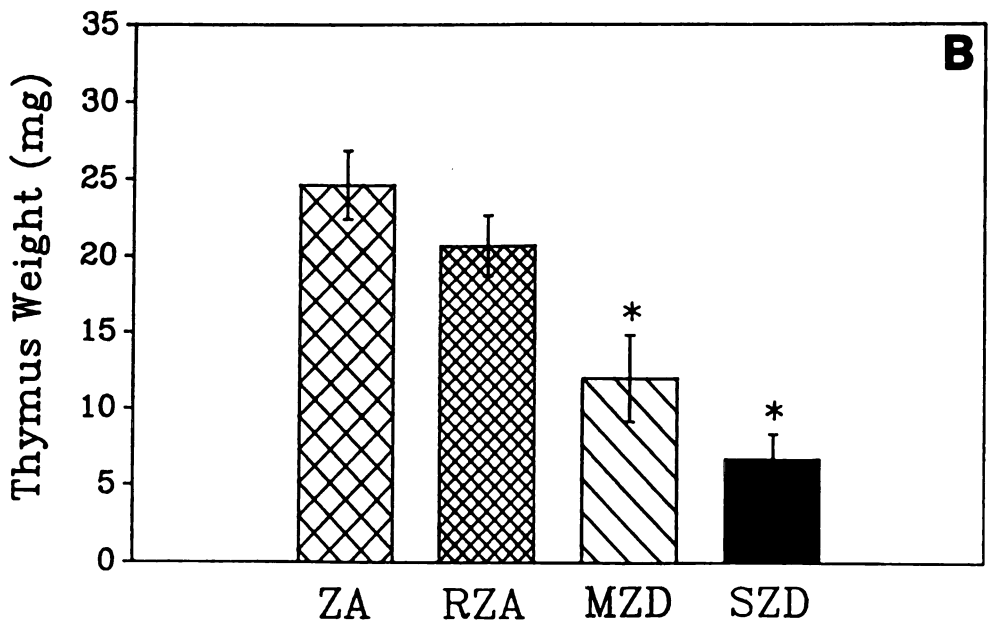

B
Thymus Weight (mg)
35
30
25
20
15
10
5
0
*
*
ZA
RZA
MZD
SZD

Figure 2: Analysis of the zinc content of sera and tissues of mice from the ZA, RZA, MZD, and SZD groups at day 27. (A) Serum from individual mice was evaluated for zinc content using atomic absorption spectroscopy. (B) Aliquots ($1x10^7$ cells) of nucleated BM cells from individual mice in each of the four dietary groups were digested and analyzed for zinc content via the ICP/AES method. (C) Tibia and femurs devoid of marrow, and the upper left liver lobe from mice were digested and analyzed by ICP/AES analysis. ZA, n=8; RZA, n=5; MZD, n=7; SZD, n=7. Data are expressed as mean$\pm$SD. * Denotes data significantly different from control (ZA) at $P<0.05$.

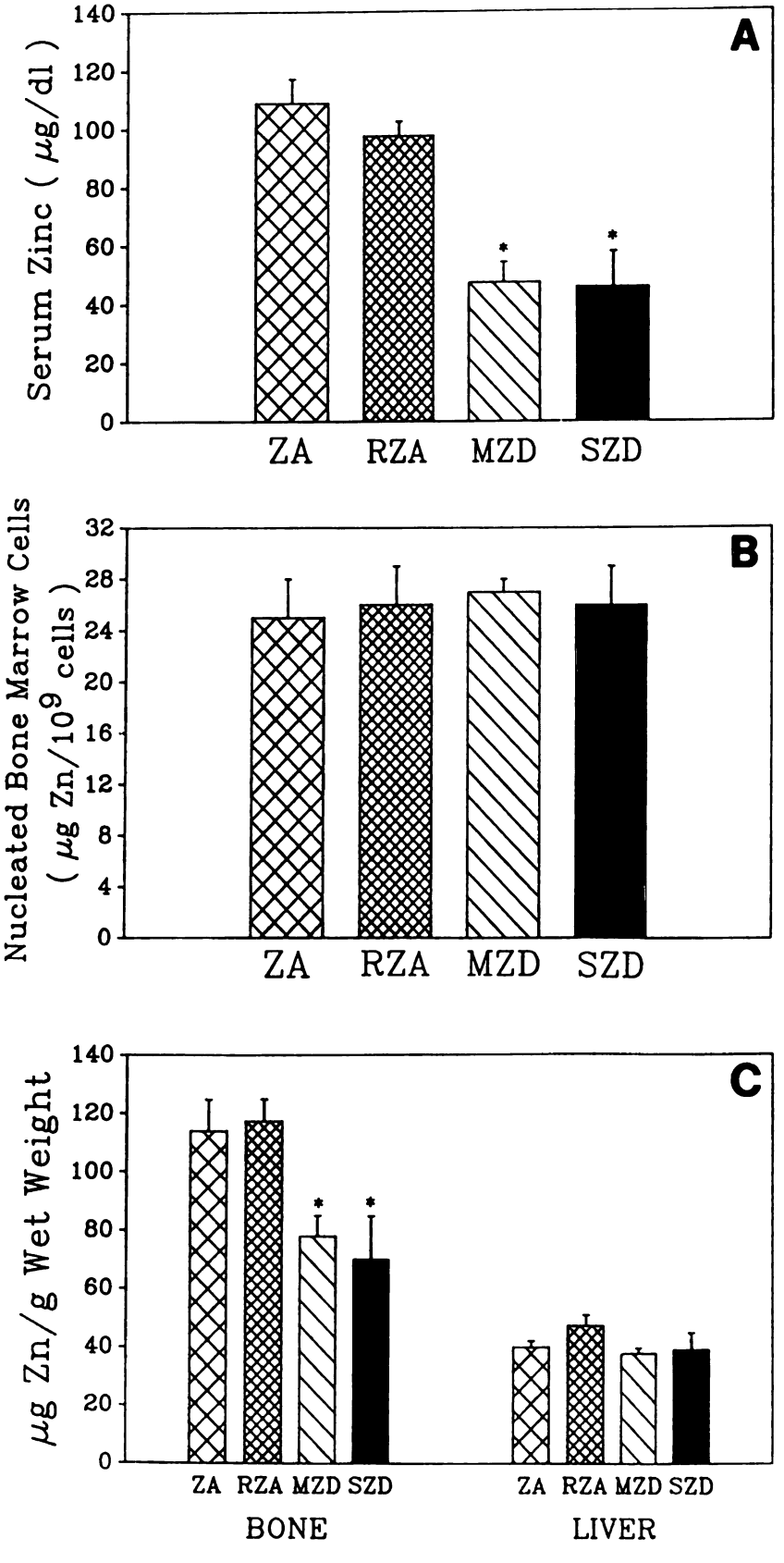

A
Serum Zinc (μg/dl)
140
120
100
80
60
40
20
0
*
*
ZA
RZA
MZD
SZD
B
Nucleated Bone Marrow Cells
(μg Zn/10^9 cells)
32
28
24
20
16
12
8
4
0
ZA
RZA
MZD
SZD
C
μg Zn/g Wet Weight
140
120
100
80
60
40
20
0
*
*
ZA RZA MZD SZD
BONE
ZA RZA MZD SZD
LIVER

Figure 3: Schematic representation of stages of B-cell development in the BM of murine system as proposed by Hardy *et al.*, 1991. Acquisition and/or loss of surface maturation markers, progression through Ig gene rearrangement, and the growth requirement (not shown) were utilized to identify different subpopulations in the B-cell compartment of the murine BM in this diagram.

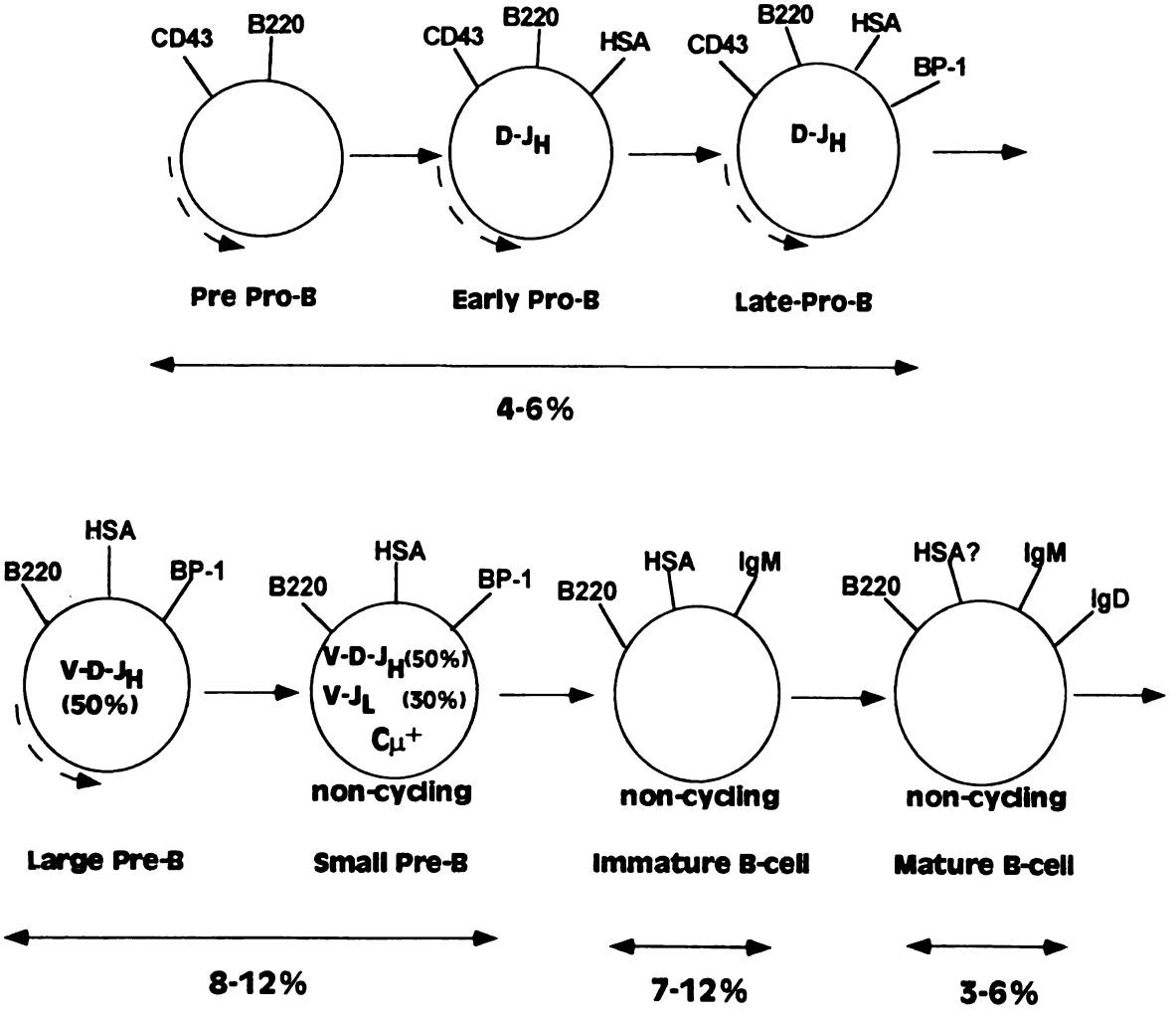
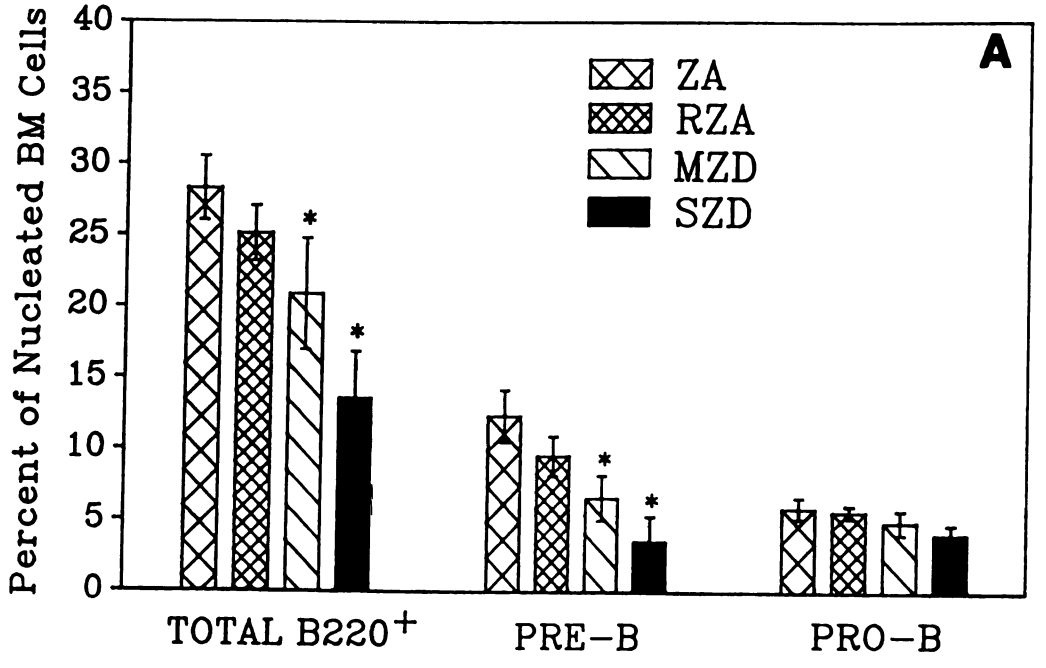

CD43
B220
CD43
B220
HSA
CD43
B220
HSA
BP-1
D-J$_H$
D-J$_H$
Pre Pro-B
Early Pro-B
Late-Pro-B
4-6%
HSA
B220
BP-1
V-D-J$_H$ (50%)
HSA
B220
BP-1
V-D-J$_H$(50%)
V-J$_L$ (30%)
Cμ+
HSA
IgM
B220
HSA?
IgM
IgD
B220
non-cycling
non-cycling
non-cycling
Large Pre-B
Small Pre-B
Immature B-cell
Mature B-cell
8-12%
7-12%
3-6%

Figure 4: Effects of ZD on phenotypic distribution of early B-cells of the marrow. **(A)** A three color phenotyping of BM B-cells using anti-B220-PE, anti-CD43/S7-FITC, anti-IgM-biotin was used to identify precursor and pro-B cells. Total B-cells consist of all the $B220^+$ cells, pro-B cells are defined as ($B220^+S7^+IgM^-$) and pre-B cells are $B220^+S7^-IgM^-$). **(B)** The phenotypic distribution of early pro-B cells ($B220^+S7^+6C3^-$) and late pro-B cells ($B220^+S7^+6C3^+$) using a second set of three color phenotyping. For these experiments ZA, n=7; RZA, n=5; MZD, n=9; SZD, n=8. Data are shown as mean$\pm$SD being representative of 3 separate experiments. * Denotes data significantly different from ZA group at $p<0.05$.


A
ZA
RZA
MZD
SZD
Percent of Nucleated BM Cells
40
35
30
25
20
15
10
5
0
*
TOTAL B220+
PRE-B
PRO-B

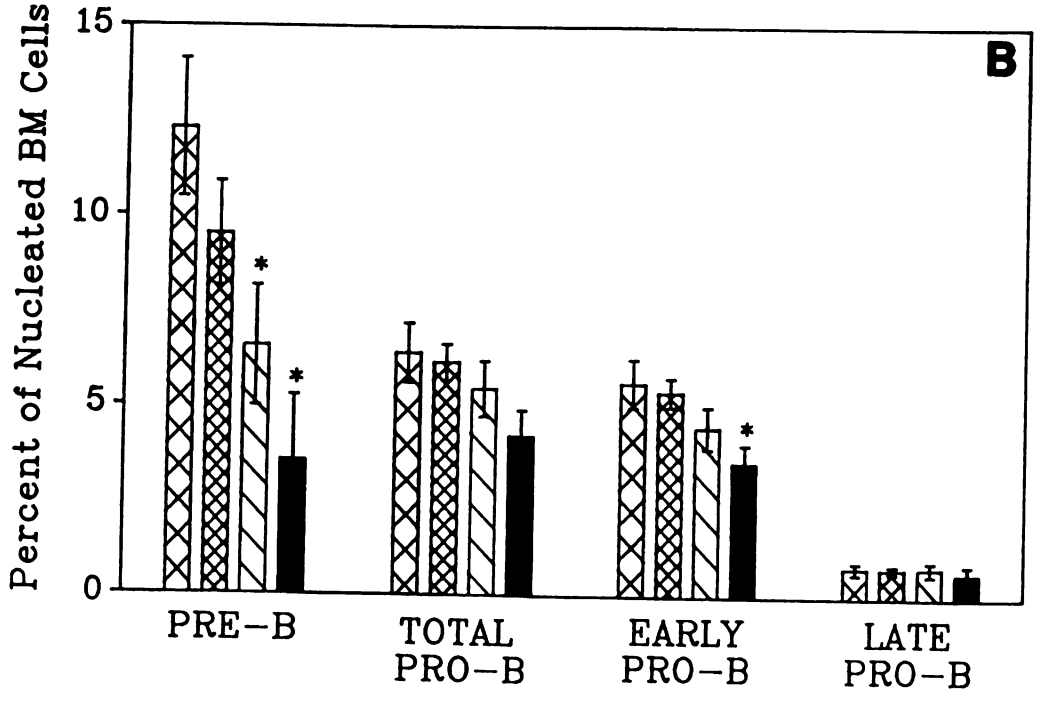
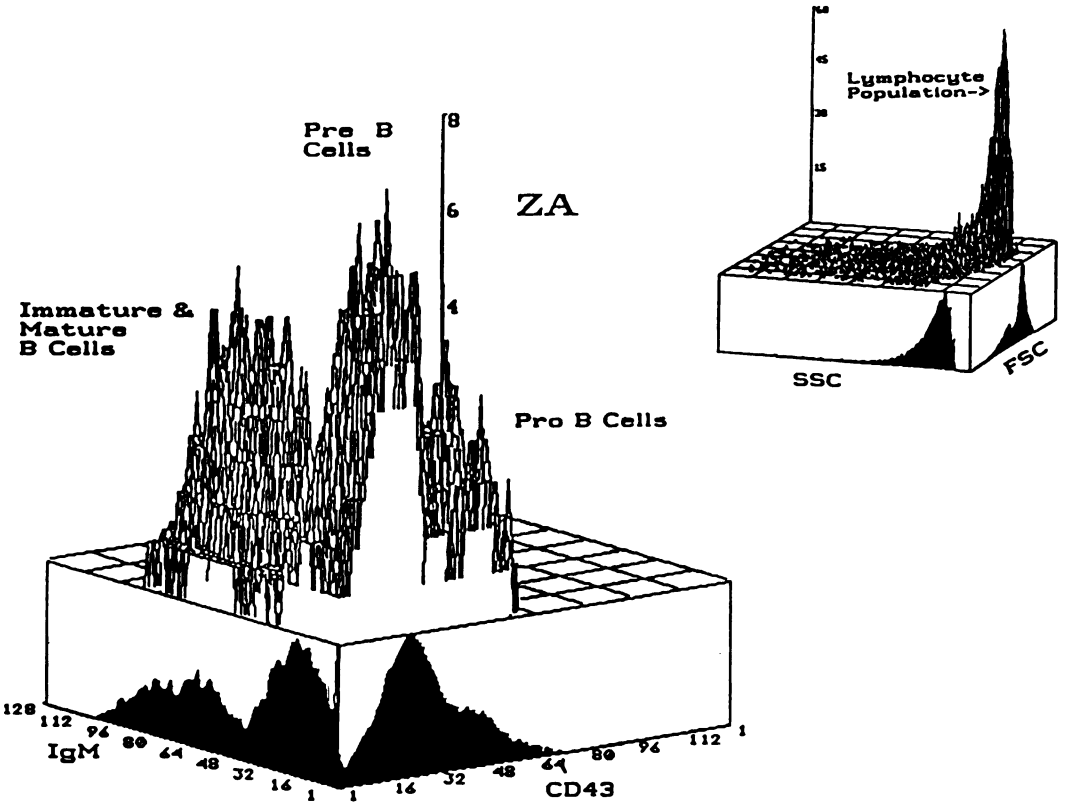

B
Percent of Nucleated BM Cells
15
10
5
0
*
PRE-B
TOTAL PRO-B
EARLY PRO-B
LATE PRO-B

Figure 5: Three-dimensional presentation of flow cytometric scatter profiles of $B220^+$ gated nucleated BM cells from a ZA (**Top panels**) and a SZD (**Bottom panels**) mice after a 27-day dietary period. **Left panels** demonstrate the 2-color (IgM vs CD43) cytogram scatter profiles of three major subpopulations of the B-cell compartment (Pro, Pre, and immature/mature B-cells) in the marrow of a ZA or a SZD mouse. **Right panels** illustrate the light scatter profiles of lymphocytes gated $B220^+$ population in the BM of a ZA and a SZD mice, respectively. Forward scatter (FSC) is indicative of cell size while side scatter (SSC) indicates cell granularity. Data are from a ZA (control) and a SZD mouse representative of seven to eight mice in each dietary group, respectively.


Pre B
Cells
8
6
4
ZA
Immature &
Mature
B Cells
Pro B Cells
Lymphocyte
Population->
SSC
FSC
128
112
96
80
64
48
32
16
1
IgM
CD43

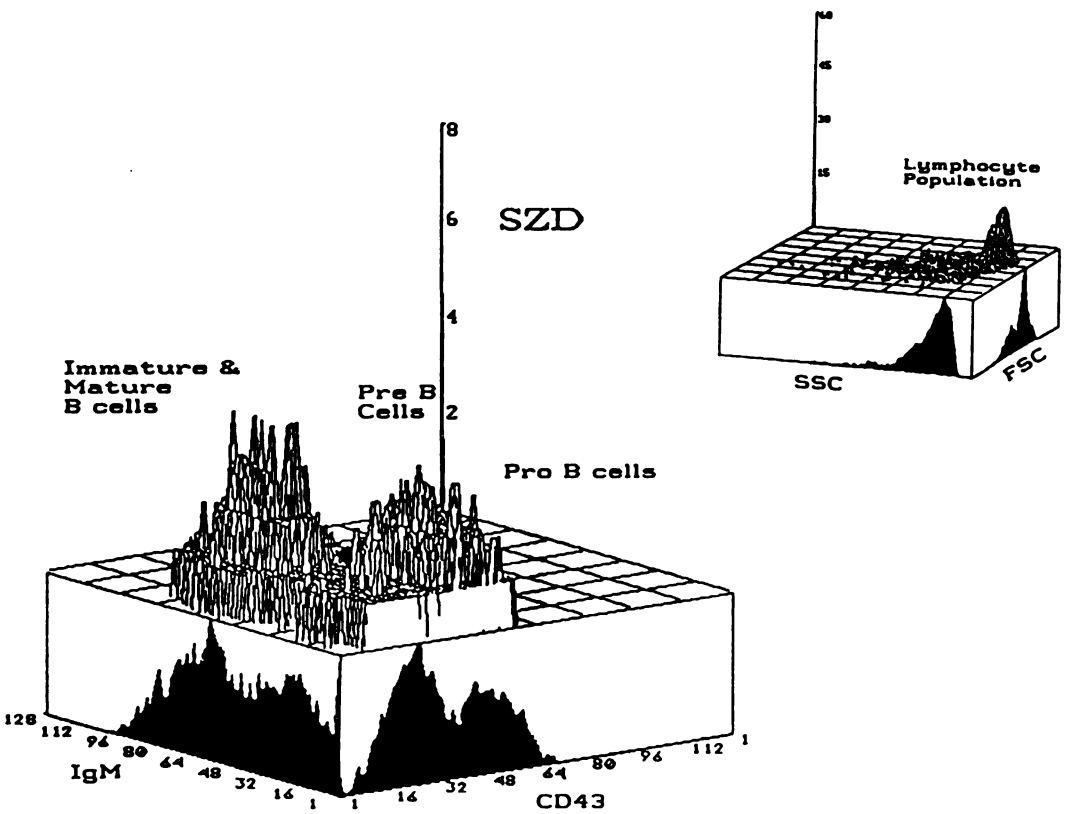

8
6
4
2
SZD
Immature &
Mature
B cells
Pre B
Cells
Pro B cells
Lymphocyte
Population
SSC
FSC
128
112
96
80
64
48
32
16
1
IgM
CD43

Figure 6: Expression of precursor (Pre-B) and progenitor (Pro-B) B-cells as a proportion of BM B-cell compartment for ZA, RZA, MZD, and SZD mice at day 27. Data is from the experiment presented in Figure 4A. ZA, n=7; RZA, n=5; MZD, n=9; SZD, n=8.

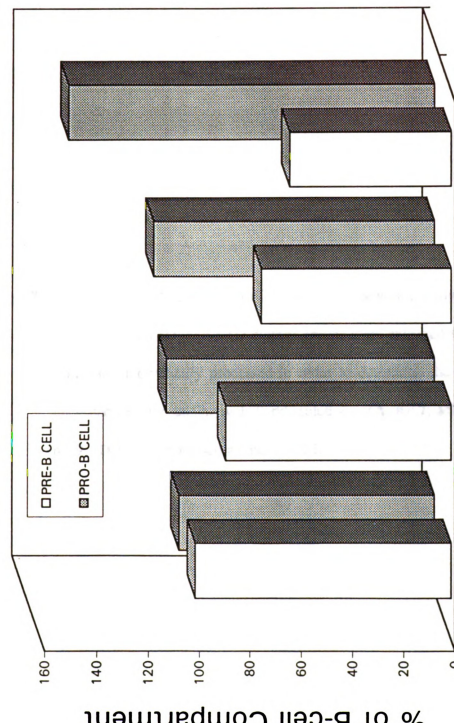
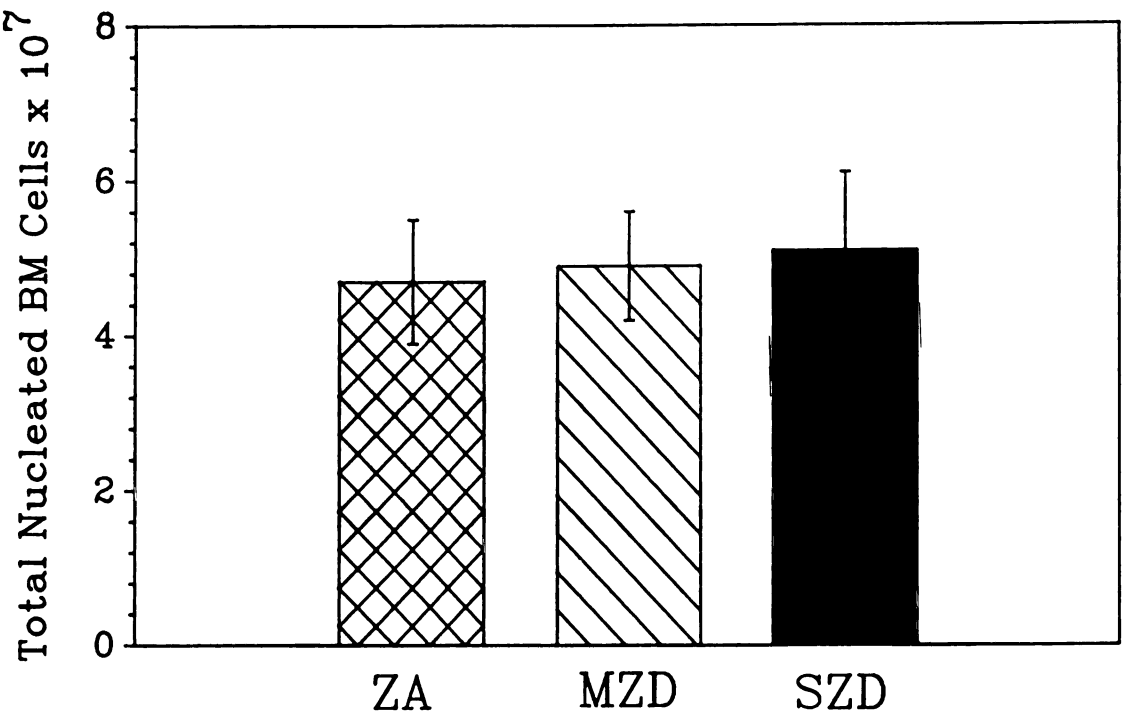

% of B-cell Compartment
160
140
120
100
80
60
40
20
0
PRE-B CELL
PRO-B CELL
ZA
RZA
MZD
SZD

Figure 7: Evaluation of BM cellularity for 27 day of a dietary zinc study. Marrow from mice in ZA, MZD, and SZD dietary groups were extruded from femurs and tibias and individually processed in order to determine the total number of nucleated cells by staining with Turk's solution. ZA, n=7; MZD, n=9; SZD, n=9. Data are expressed as mean $\pm$ SD.


Total Nucleated BM Cells x 10^7
8
6
4
2
0
ZA
MZD
SZD

CHAPTER FOUR

Role of Glucocorticoids in the Suppression of B-Lymphopoiesis During Zinc Deficiency

Abstract

Previous investigations from this laboratory have provided strong evidence that the rapid thymic atrophy and bone marrow (BM) B-lineage lymphopenia associated with zinc deficiency in mice correlated with chronically elevated levels of plasma corticosterone (CS). Furthermore, removal of endogenous CS by adrenalectomy provided significant thymic protection against zinc deficiency in adrenalectomized mice (ZD Adr) as compared to zinc deficient non-adrenalectomized controls (ZD sham). However, it was not clear whether adrenalectomy in ZD mice would also protect BM cellularity, particularly the B-cell compartment. To address the role of endogenous glucocorticoid (GC) elevation on B-lymphopoiesis in ZD mice, a zinc dietary study using adrenalectomized and non-adrenalectomized mice was conducted. However, due to the special care and handling required of adrenalectomized mice, a typical 30-day dietary study was lengthened to 8 weeks. As a result, mice in the ZD group were only moderately deficient. Plasma CS levels showed a six-fold increase in ZD shams compared to ZD Adr mice, indicating the induction of the stress axis by ZD and successful removal of GCs via adrenalectomy. Consistent with the earlier report from this laboratory, removal of adrenal glands provided substantial protection of the thymus of ZD mice, whereas ZD non-adrenalectomized group exhibited a 28% thymic atrophy compared to ZA sham. Interestingly, two color flow cytometric analysis of BM B-lymphocytes in ZD Adr indicated a complete protection of early developing ($B220^+sIg^-$) and immature ($B220^+IgM^+IgD^-$) B-lineage populations, as their proportions were analogous to those of ZA sham group. As expected, ZD sham operated mice exhibited significant depletion in early

(57%) and immature (50%) B-cells comparing with ZA shams. However, there was no significant change in the mature ($B220^{+}IgM^{+}IgD^{+}$) B-cell compartment in either of ZD Adr or ZD sham operated mice. Taken together, the data confirms the selective sensitivity of developmental stages of BM B-lymphocytes to the effects of ZD, as was demonstrated in previous chapters. More importantly, it suggests a role for chronic elevation of CS during ZD in the alteration of B-lineage lymphopoiesis in the murine system.

Introduction

Malnutrition including zinc deficiency has been shown to exert a rapid and deleterious effect on host defense in both humans and animals (Fraker *et al.*, 1986; Fraker *et al.*, 1993; Kuvibidila *et al.*, 1993; Prasad, 1995). Earlier studies from this lab have shown a significant alteration in both antibody and cell mediated responses during the course of ZD (Fraker *et al.*, 1986; Fraker *et al.*, 1993). Nevertheless, splenic T and B-cells from ZD mice were responsive to different mitogenic stimuli indicating normal functional capacity (Cook-Mills and Fraker, 1993a). Further studies indicated rapid thymic atrophy as well as lymphopenia in zinc deficient mice (DePasquale-Jardieu and Fraker, 1979; Fraker *et al.*, 1982; King and Fraker, 1991). Thus, it was clear that part of the reduction of host defense in ZD was due to the reduced number of leukocytes actively participating in immune responses. Data presented in previous chapters (2,3) clearly indicated that ZD profoundly affected lymphopoiesis, in particular B-lymphopoiesis, in the marrow of mice. The deleterious effects on B-cell development were mainly observed in precursor and immature B-cells, leaving the progenitor and mature B-cells relatively unaffected. However, the mechanism(s) underlying these alteration was still unknown.

It is well documented that physiological stresses such as surgery, burns, infections, and malnutrition are associated with a two to five fold increase in circulating plasma GC levels (Maldonado *et al.*, 1991; Barone *et al.*, 1993; Hermann *et al.*, 1994; Fraker *et al.*, 1995). In fact, there are studies in which the role of elevated levels of endogenous GCs in thymic atrophy and lymphopenia in ZD has been suggested (Quarterman, 1973; Quarterman

and Hamphires, 1979; DePasquale-Jardieu and Fraker, 1979; 1980). A few years ago, this laboratory demonstrated that ZD, possibly through the activation of the stress axis, led to the chronic elevation of GC from a basal level (10-20 μg/dl) to a higher level (100-120 μg/dl) which was shown to be immunosuppressive (DePasquale-Jardieu and Fraker, 1979). In addition, a series of *in vivo* studies were performed using implanted CS pellets in mice to chronically deliver levels of CS (32 to 94 μg/dl) similar to the levels detected in ZD (Garvy *et al.*, 1993a). Interestingly, a short exposure (3 days) to these levels of CS resulted in thymic atrophy and significant reduction (~70%) of BM early developing B-cells ($B220^+$, sIg^-) (Garvy *et al.*, 1993a). In separate studies, this lab also demonstrated that removal of endogenous CS via adrenalectomy provided complete thymic protection against atrophy in ZD Adr compared with ZD sham (DePasquale-Jardieu and Fraker, 1980). Considering the above observations, one might hypothesize that if immature B-cells are as sensitive as immature thymocytes to chronically elevated levels of GC, removal of circulating corticosteroid by adrenalectomy should also provide BM protection against ZD as it did to the thymus. To investigate this question, mice were either adrenalectomized or sham operated and after 7-10 days of recovery they were started on a dietary zinc study. In a typical diet study, mice are normally housed in hanging stainless steel cages with open-wire mesh bottoms to prevent zinc contamination from their feces (Cook-Mills and Fraker, 1993a). However, to keep these vulnerable mice as warm and protected as possible, the metal cages were replaced with EDTA washed polycarbonate cages with hardwood chips bedding. While these conditions protected the mice from additional stress, they increased the possibility of zinc recirculation between the mice and their surrounding environment. For these reasons,

the diet study was lengthened to eight weeks resulting in only moderate degree of deficiency among mice in the ZD group. Results presented herein confirm the role of adrenalectomy in protection of the thymus against atrophy in ZD, as was demonstrated in an early study by DePasquale-Jardique and Fraker (1980). More significantly, removal of the CS provided substantial protection of B-lymphopoiesis in the marrow of ZD mice as well.

Materials and Methods

Mice and Diet:

Young adult A/J female mice (4-5 weeks old) obtained from the Jackson Laboratory (Bar Harbor, Maine) were used in this study. Mice were housed in transparent polycarbonate cages with woodchip bedding and maintained in a temperature (72-74°F) and humidity (45-50%) controlled facility on a 12 hr/12 hr light/dark cycle (lights on 7 a.m.). Mouse chow and acidified water were freely available and 5-7 days of climate adjustment was allowed prior to the surgery. Following surgery, at least 7-10 days were allowed for healing before starting the dietary study. In addition, to maintain the mice as warm and comfortable as possible, hanging stainless steel cages were not used. Instead, the mice were housed in pairs in EDTA washed polycarbonate cages provided with 4N HCl washed feed jars and water bottles. These conditions lengthened the period of dietary study to about 8 weeks resulting in only a moderate degree of deficiency. To initiate the dietary study, mice were subdivided into four dietary groups: zinc adequate adrenalectomized (ZA Adr), zinc adequate sham operated (ZA sham), zinc deficient adrenalectomized (ZD Adr) and zinc deficient sham operated (ZD sham). Mice in each group were fed ad libitum a biotin-fortified egg white containing either deficient (~0.5 ppm) or adequate (~30 ppm) levels of zinc (see Table 1 in Chapter 3). Adrenalectomized and non-adrenalectomized mice were maintained on physiological saline (0.9% NaCl) and deionized distilled drinking water, respectively. The diet study was terminated using criteria for moderately zinc deficient mice such as moderate changes in coat color, texture, eye, tail and skin along with diminished food consumption and body weights.

Adrenalectomy:

Mice that were to be adrenalectomized were anesthetized with methoxyflurane (Metafane, Pitman-Moore, Inc., Mundelein, IL) using a nose cone and received bilateral dorsal incisions through the skin and muscle wall at the level of the last rib. To maintain consistency in the operational procedure, the adrenal gland on the left side was always removed first and the right adrenal gland was removed subsequently. The incision was closed with 6-0 ethilon sutures (Ethicon Inc., Somerville, NJ). Sham operated mice (controls) were exposed to the same surgical procedure except the adrenal glands were left intact. All surgical procedures were done using sterile techniques. Following the surgery, all of the mice received 0.3 ml subcutaneous injections of Buprenex (2.5 ng/ml) in Lactated Ringers solution followed by two to three more injections at 12-hr intervals to minimize post-surgical pain and dehydration. Adrenalectomized and sham operated (non-adrenalectomized) mice were then placed individually in cages with sterile, autoclaved bedding material. A recovery period of 7-10 days was allowed before the initiation of the zinc dietary study. The success of the operation was verified via visual inspection for any trace amount of adrenal glands and by measurement of the plasma CS levels at the time of sacrifice.

Blood Collection and Plasma Corticosterone and Zinc Assays:

To prevent elevation of endogenous corticosterone and obtain it at the lowest levels during the diurnal cycle, mice were bled within 90 seconds of disturbing the cages between 8-9 a.m. Blood samples collected from the subclavian arteries of anesthetized mice from all four dietary groups were processed individually in acid washed heparinized microtubes. A

spectrofluorometric method for measuring corticosterone in small volume of plasma (Garvy *et al.*, 1993a) was utilized. Briefly, CS was extracted from 30 µl of plasma by addition of 600 µl dichloromethane (Aldrich,Milwaukee,WI). After vigorous shaking and centrifugation, the aqueous layer (top phase) was discarded. Then the solvent layer (bottom phase) was treated with 0.1N NaOH, mixed well and centrifuged. The organic phase (bottom layer) was then used for the fluorescence development. A volume of 200 µl of a pre-fluorescence mixture consisting of three parts concentrated sulfuric acid (Aldrich,Milwaukee,WI) and one part absolute ethanol (200 proof) was added to the organic phase. To develop the fluorescence, samples were then vigorously shaken for 2 min., centrifuged and the solvent layer (top phase) was discarded by aspiration. After 30 min. incubation of the acid layer at room temperature, the fluorescence intensity was determined on a spectrofluorometer (Perkin Elmer, model 650-40) at 475 nm excitation wavelength and 525 nm emission wavelength. Standards containing 10-75 ng CS (Sigma, St.Louis, MO) were carried through the same procedure resulting in a linear relationship between the fluorescence intensity and the standard concentrations used in this study. Addition of known amount of standards to the plasma samples of known concentrations resulted in 85% to 95% recovery by this method.

For zinc analysis, plasma samples were diluted 1:10 in 1% HCl and analyzed immediately by flame atomic absorption spectrophotometry (Varian AA-20 Plus, Mulgrave, Victoria, Australia) at 213.9 nm with deuterium background correction. A standard curve was established using concentrations of zinc ranging from 0.1 to 1 ppm (µg Zn/ml) prepared from an ultra-pure standard (Sigma, St. Louis, MO). Addition of known concentrations of

zinc to plasma samples (standard addition) resulted in greater than 90% recovery of zinc by this method.

Preparation of Cell Suspension:

BM cell suspensions were prepared by flushing the femoral and tibial shafts with Hepes buffered modified Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO) supplemented with 4% FBS. After removal of erythrocytes using density gradient centrifugation over Histopaque (1083) (Sigma, St. Louis, MO), the interphase was removed, washed two times and resuspended at $1x10^6$ cells/ml in cold HBSS with 2% FBS and 0.1% sodium azide and kept on ice for phenotypic analysis.

Antibodies:

Biotin-conjugated rat anti-mouse B220 (RA3-6B2) prepared in house, sheep anti-mouse biotinylated IgD (γ chain specific) (The Binding Site, San Diego, CA), and dichlorotriazinyl amino fluorescein (DTAF) conjugated goat anti-mouse IgM (μ chain specific) from the Jackson Labs (West Grove, PA) were used in this study. For biotinylated antibodies, streptavidin conjugated phycoerythrin (AV-PE) fluorochrome, purchased from Vector (Burlingame, CA), was used to detect the biotin.

Immunofluorescence Staining:

Labeling of cell surface antigens was performed by incubating one million cells for 30 min. on ice with 150 μl of appropriately diluted antibody combinations. The antibody

combinations were either biotin-anti-B220 and DTAF-anti-IgM to detect early B-cells versus immature/mature B-Cells, or DTAF-anti-IgM and biotin-anti-IgD for the detection of immature and mature B-cells. Cells stained with biotinylated antibodies were washed once and incubated for another 30 min. with streptavidin conjugated phycoerythrin for developing fluorescence. The negative controls were unstained cells, cells stained with secondary antibody (AV-PE), and cells from tissue negative in expression of cell surface antigens (eg., thymocytes).

Flow Cytometry:

Two color FACS analysis of stained cells was performed on a Becton Dickinson Vantage flow cytometer linked to a HP consort 32 computer system on which multiparameter data were collected and analyzed using LYSIS II software. The flow cytometer was equipped with an ILT laser exciting at 488 nm. Electronic compensation was used for color correction of DTAF and PE Fluorochromes. DTAF emission was collected at 530±15 and PE emission was collected at 575±13. Singlet cells were gated by light scatter (forward vs side scatter) to exclude debris and cell aggregates. Data from 10,000 cells were collected in list mode.

Statistical Analysis:

All data were analyzed by the Kruskal-Wallis nonparametric test and the analysis of variance (ANOVA) for parametric data in order to identify significant differences between dietary groups (Daniel, 1987). A Tukey's post-hoc comparisons was performed when

appropriate (Daniel, 1987). Differences were considered statistically significant at $P<0.05$. All data are presented as the mean $\pm$ SD where *n* is 6 to 8 mice unless otherwise indicated.

Results

Effects of Adrenalectomy on Body Weight and Thymus Integrity of Zinc Deficient Mice:

The change in body weights of the four different dietary groups obtained at different time points during the diet study are shown in Table 1. As indicated in Table 1, the body weights in ZD Adr mice were not significantly different from their corresponding control group, ZD sham, throughout the diet study. However, by the end of the dietary study ZD adrenalectomized and sham operated mice weighed significantly less than adrenalectomized and sham operated ZA fed groups. In this case, ZD Adr mice weighed 86% and ZD sham 79% of their corresponding controls, ZA Adr and ZA sham mice.

In terms of thymus integrity, the thymus weight (Figure 1) of the ZD sham mice decreased significantly, being 72% of control sham operated ZA fed mice. The degree of atrophy was in accordance with the values obtained from mice moderately affected by zinc deficiency in the previous chapters (2,3). More importantly, the thymus weights of the ZD Adr group remained at about 90% of the ZA Adr and 1.5 fold greater than that in ZD shams, indicating significant protection against the effects of ZD (Figure 1).

Effects of Adrenalectomy on Plasma CS and Zinc levels in Mice on Zinc Deficient Diet:

upon termination of the dietary study (day 56), mice from each of the four dietary groups were bled and their plasma CS was determined by a fluorometric assay (Garvy *et al.*, 1993a). As shown in Figure 2, the mean plasma CS level in ZD sham operated mice was 2.6

and 6 fold greater than that in the control ZA sham and ZD Adr groups, respectively. Further, the plasma CS levels in ZD Adr and ZA Adr groups remained at 2.8-4.1 μg/dl, while the levels in ZA sham operated mice was elevated slightly to 6.6 μg/dl (Figure 2).

The results of the plasma zinc levels of the mice in each group obtained at the termination of diet study are illustrated in Figure 3. Adrenalectomies appeared to have no effect on the plasma zinc levels, as no significant difference in plasma zinc level was noted between ZD Adr and ZD sham mice. Likewise, the zinc level in ZA Adr mice showed no significant change compared to the levels detected in ZA sham operated group. By contrast, plasma zinc levels in mice from ZD fed adrenalectomized and sham operated groups were moderately declined (21-28%) compared to the control ZA fed adrenalectomized and sham operated mice, respectively (Figure 3).

B-lymphopoiesis in Zinc Deficient Adrenalectomized Mice:

To determine the effects of adrenalectomy or removal of GC on B-cell development in ZD mice, fluorescently labeled antibodies directed against major B-cell surface markers, B220, IgM and IgD, were used, and flow cytometric analysis was performed. Using this approach, the proportion of early ($B220^+Ig^-$), immature ($B220^+IgM^+IgD^-$), and mature ($B200^+IgM^+IgD^+$) B-cell subcompartments in the marrow of mice in each of the four dietary groups were determined (Figure 4). In Figure 4, it is clear that ZD had a profound effect on cells of the B-lineage at the early and immature stage of development as shown in previous studies (Chapters 2,3). The proportion of early developing B-cells ($B220^+Ig^-$) in ZD sham mice indicated 57-70% depletion compared to that of ZA sham and ZD Adr groups,

respectively. Similarly, immature ($B220^+IgM^+IgD^-$) B-cell declined significantly (57-65%) in the marrow of ZD sham operated group when compared to the ZA sham and ZD Adr mice. However, the more mature ($B220^+IgM^+IgD^+$) B-cell subset showed marginal but not statistically significant reduction in ZD sham operated mice. Interestingly, adrenalectomized mice maintained on the ZD diet (ZD Adr) demonstrated complete protection in their B-cell subpopulations. The immature ($B220^+IgM^+IgD^-$) and mature ($B220^+IgM^+IgD^+$) B-cell subsets in the ZD Adr mice exhibited no depletion, as their proportions were analogous to those found in ZA Adr and sham operated mice. Likewise, the early B-cells in the marrow of ZD Adr group were not affected by the deficiency, showing a moderate accumulation (+23% to +39%) compared to that in ZA Adr and ZA sham controls, respectively (Figure 4).

Discussion

The disruption of B-cell development in the marrow of ZD mice resulting in lymphopenia, along with chronic release of known immunosupressor, GCs, during zinc deprivation suggested a role for GC for these observations. In the light of an early study (DePasquale-Jardieu and Fraker, 1980), in which removal of GC via adrenalectomy provided complete protection of the thymus of ZD mice against atrophy, speculation on the role of GC with respect to B-cells was intensified. To investigate the role of CS (the predominant form of GCs in murine system) in lymphopoiesis, adrenalectomy was performed to essentially remove GC from the circulation. Using this approach, it was possible to ascertain how much of the alteration in lymphopoiesis was due to suboptimal zinc levels versus the chronic levels of CS that are produced in later stages of the deficiency in mice. Adrenalectomy has been previously shown by this laboratory as a convenient way for removal of GC (DePasquale-Jardieu and Fraker, 1980). Furthermore, the use of this technique by many other investigators for the characterization of the potential role of different adrenal hormones including GCs (Dunn, 1988; Sloviter *et al.*, 1989; Jerrells *et al.*, 1990; Mitchell and Meaney, 1991; Rinner *et al.*, 1992; Kitson *et al.*, 1994; Dhabhar *et al.*, 1995) added more reasonings for the selection of this technique. It should be noted that although adrenalectomy also removes mineralocorticoids, the substitution of isotonic sodium chloride for drinking water has been shown to compensate for the loss of this hormone (Saksi and Epstein, 1990). An alternative to adrenalectomy would be drugs which block glucocorticoid receptors or release of GCs.

Such treatment would require continuous delivery to the already stressed dietary mice throughout the experiment (at least four weeks), which is impractical.

Just as zinc influences the hormonal secretion, particularly from the adrenal glands, changes in the concentrations of adrenal hormones (eg., GCs) have been shown to affect zinc concentrations. Early studies have indicated that adrenalectomy or adrenocortical insufficiency (eg., hypopituitarism) have been accompanied by increased serum zinc concentrations, decreased urinary zinc excretion, and increased retention of zinc in several tissues (Flynn *et al.*, 1973; Henkin, 1974). Thus, it was of interest to measure zinc levels of all mice, particularly those in adrenalectomized groups, to monitor any possible variation in zinc levels due to adrenalectomy as well as suboptimal zinc intake. As shown in Figure 3, the results indicated a moderate but statistically significant decline in the zinc levels of ZD Adr and ZD sham groups indicating the expected depletion of zinc in moderate deficiency. On the other hand, the plasma zinc levels in ZD Adr group showed no significant variations compared to ZD sham operated group. Likewise, no significant change in plasma zinc levels of ZA Adr compared to that in ZA sham group was observed. These data suggest that adrenalectomy or removal of CS from the circulation had no apparent effects on serum zinc distribution as opposed to what was indicated by earlier studies (Flynn *et al.*, 1973; Henkin, 1974).

Although moderately zinc deficient, the mice in ZD sham operated group had 6 fold higher plasma CS levels compared to the levels detected in ZD Adr group. This indicates the activation of the hypothalamus-pituitary-adrenocortical axis (stress axis) in ZD sham mice as reported in the literature (DePasquale-Jardieu and Fraker, 1979; 1980; Quarterman and

Humphries, 1979; Prasad, 1985;) at even moderate stages of the deficiency. Furthermore, the low levels of CS in adrenalectomized mice compared to non-adrenalectomized mice (sham operated) were indicative of proper adrenalectomy or nearly complete elimination of CS via adrenalectomy, and the lack of detectible adrenal tissue at the time of sacrifice supported this.

Examination of the thymuses of ZD group revealed that in spite of moderate induction of ZD in this study, the thymus of ZD sham operated mice were involuted 28% to 33%, compared to the thymus weights of the ZA shams and ZD Adr mice. This observation, along with the similarity between the thymic weights of ZD Adr and ZA Adr groups, strongly suggested a role for GC in thymic atrophy, as the elimination of this hormone significantly protected the thymus from atrophy (DePasquale-Jardieu and Fraker, 1980; Jerrells *et al.*, 1990).

The major question to be addressed in this study was the degree to which adrenalectomy had protected the development of cells of B-lineage in the ZD Adr mice. As shown in Figure 4, B-cell subpopulations whose susceptibility to the effects of ZD has been shown herein and in the previous chapters (2 and 3) were now completely protected with adrenalectomy. In ZD sham operated mice where CS was elevated, early and immature B-cell subcompartments exhibited significant depletion in accordance to the previous findings (King *et al.*, 1995). However, adrenalectomy appeared to provide substantial protection to the developing B-cells in ZD mice. This was demonstrated by the presence of all the BM B-cell subpopulations evaluated in this study in proportions similar to those detected in ZA Adr and ZA sham operated mice. Thus, suboptimal zinc may not be directly affecting the marrow's ability to produce new lymphocytes during moderate deprivation. Secondary effects of zinc

deficiency, especially the elevation in production of glucocorticoids, may be significantly reducing lymphopoiesis.

As stated earlier, *in vivo* exposure of mice to the levels of CS induced by ZD were shown to be sufficient to alter B-lymphopoiesis, particularly in early and immature stages of B-cell development (Garvy *et al.*, 1993a). Furthermore, sensitivity of these populations to short exposure (8 hrs) of synthetic (dexamethasone) and natural (cortisol, corticosterone) GC *in vitro* was also demonstrated by their substantial depletion in the BM and their accumulation in the apoptotic region of DNA histogram evaluated by flow cytometry (Garvy *et al.*, 1993b; Voetberg *et al.*, 1994). These findings are in accordance with the more recent literature in which adrenalectomy protected the suppressive effects of GC on cells of the immune system (Kitson *et al.*, 1994; Dhabhar *et al.*, 1995). The results from this study clearly demonstrated the neuroendocrine (stress-induced CS release) regulation of the immune system suggested in the literature (Bateman *et al.*, 1989; Dantzer and Kelley, 1989; Homo-Delarche and Dardenns, 1993; Berczi, 1994).

It appears that although zinc is an essential element in many biochemical activities inside cells (Vallee and Falchuk, 1993; Cousins, 1996), the deficiency of zinc by itself may not be directly exerting suppressive effects on the cells of immune system. Instead, it is the stimulation of the stress axis with the production of GCs at elevated levels, which leads to immunosuppression in ZD (DePasquale-Jardieu and Fraker, 1979; Prasad, 1985; Hambidge *et al.*, 1986; Bunce, 1989). Thus, it seems that ZD purposely downsizes the immune system, which is one of the largest tissues and major users of nutrients in the body, to provide this limited nutrient to other vital tissues for their protection. This is achieved apparently via

activation of the stress axis and subsequent chronic release of GC in later stages of the deficiency. On the other hand, the survival of mature lymphocytes (T and B) in the course of ZD provides a fully functional defense system (Cook-Mills and Fraker, 1993a), but with lower magnitude in the periphery. Taken together, these observations identified the significant role of CS in the alteration of murine BM B-lymphopoiesis in the course of suboptimal dietary zinc intake. However, the possibility of other factors (eg., oncogenes) that might play additional mechanistic roles (eg., blocking or facilitating GC actions) can not be ruled out and needs to be investigated.

Table 1: Body weights of sham operated and adrenalectomized mice maintained on zinc adequate or zinc deficient diet during eight weeks of diet study.

Weeks on Diet	ZA Adr n=6	ZA sham n=8	ZD Adr n=7	ZD sham n=8
0	16.48 ± 0.31	16.84 ± 0.68	17.01 ± 1.22[a]	16.74 ± 1.14
2	17.18 ± 1.14	17.14 ± 1.67	17.08 ± 0.61	16.29 ± 1.25
4	18.04 ± 0.83	18.32 ± 1.2	17.50 ± 0.72	16.53 ± 1.36
6	18.66 ± 0.50	19.41 ± 1.0	17.35 ± 0.78	16.58 ± 1.96
8	19.70 ± 0.56	20.44 ± 1.22	16.90 ± 0.8[b]	16.14 ± 1.63[c]

a Data are expressed as mean ± SD of 6 to 8 mice per dietary group, and represent 2 separate experiments.

b Significantly different from ZA Adr at $P < 0.05$.

c Significantly different from ZA sham at $P < 0.05$.

Figure 1: Comparison of thymic weights of adrenalectomized and sham operated mice maintained on zinc adequate or zinc deficient diet, obtained at the terminal point of the diet study (day 56). ZA Adr, n=6; ZA sham, n=8; ZD Adr, n=7; ZD sham, n=8. Data are mean ± SD and represent two separate diet studies. *Denotes significantly different from ZA sham operated and ZD Adr mice at $P<0.05$, where comparisons were made separately .

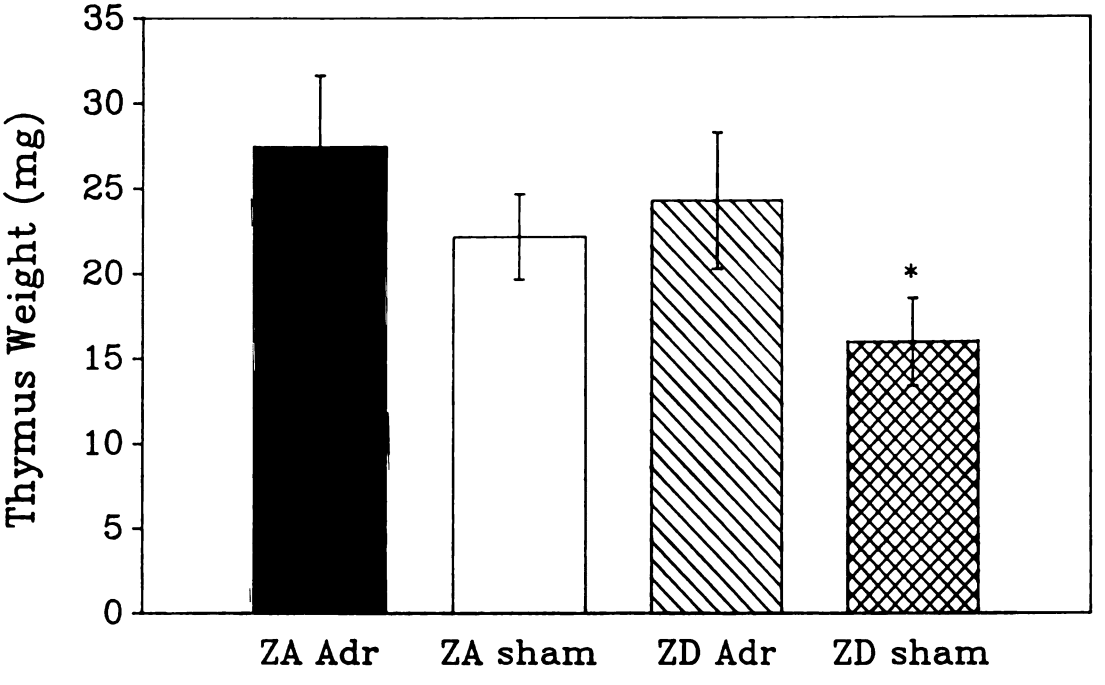

Thymus Weight (mg)
35
30
25
20
15
10
5
0
*
ZA Adr
ZA sham
ZD Adr
ZD sham

Figure 2: Plasma corticosterone levels of adrenalectomized and sham operated mice fed zinc adequate or zinc deficient diet. Upon termination of diet study (day 56), mice from each of the four dietary groups were bled and their plasma was collected individually for a fluorometric CS assay. ZA Adr, n=6; ZA sham, n=8; ZD Adr, n=7; ZD sham, n=8. Data are expressed as mean ± SD and are representative of two separate diet studies. * Denotes significantly different from ZA sham and ZD Adr groups at $P < 0.05$ in separate comparisons.

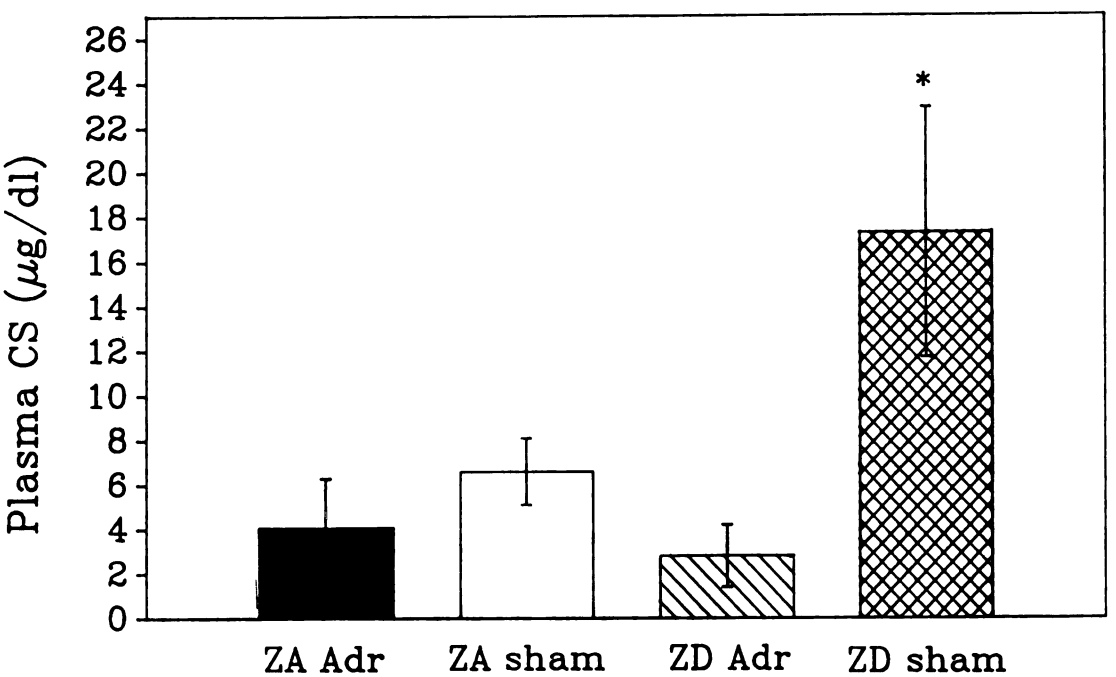

Plasma CS (μg/dl)
26
24
22
20
18
16
14
12
10
8
6
4
2
0
*
ZA Adr
ZA sham
ZD Adr
ZD sham

Figure 3: Comparison of plasma zinc levels of adrenalectomized or sham operated mice maintained on zinc adequate or deficient diet for eight weeks. Plasma zinc concentration of individual mice from each of the four dietary groups were determined via flame atomic absorption spectroscopy on the last day of diet study (day 56). Data represent mean ± SD, where n=6 to 8 mice in each dietary group. * Indicates significantly different from ZA sham at $P < 0.05$. ** Indicate significantly different from ZA Adr at $P<0.05$.

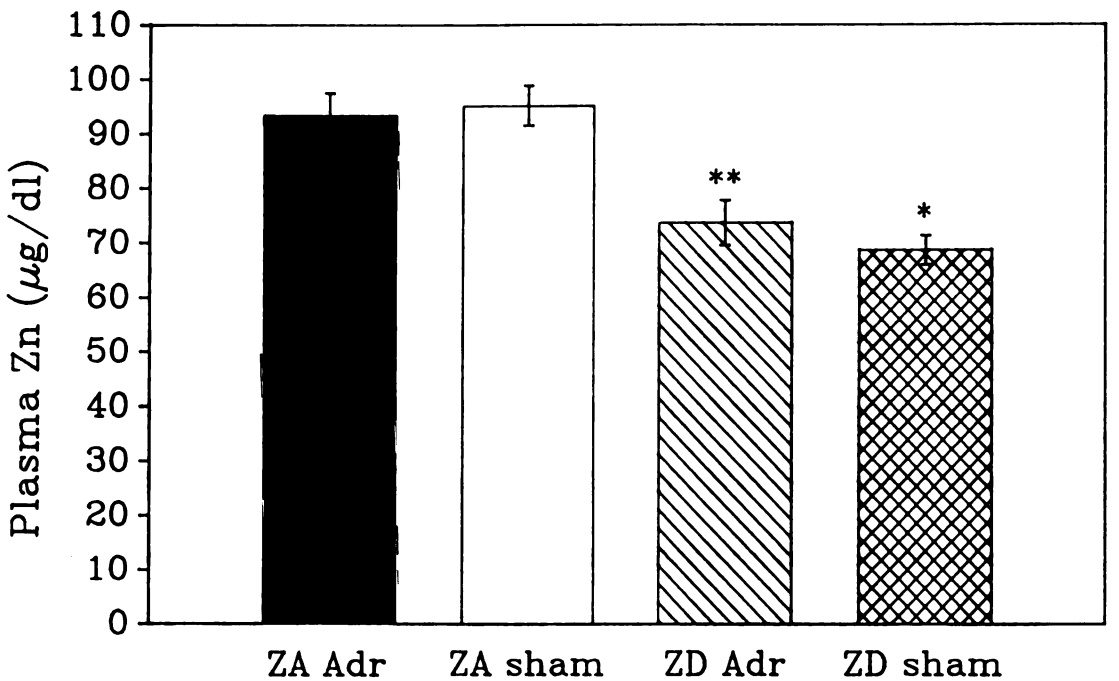
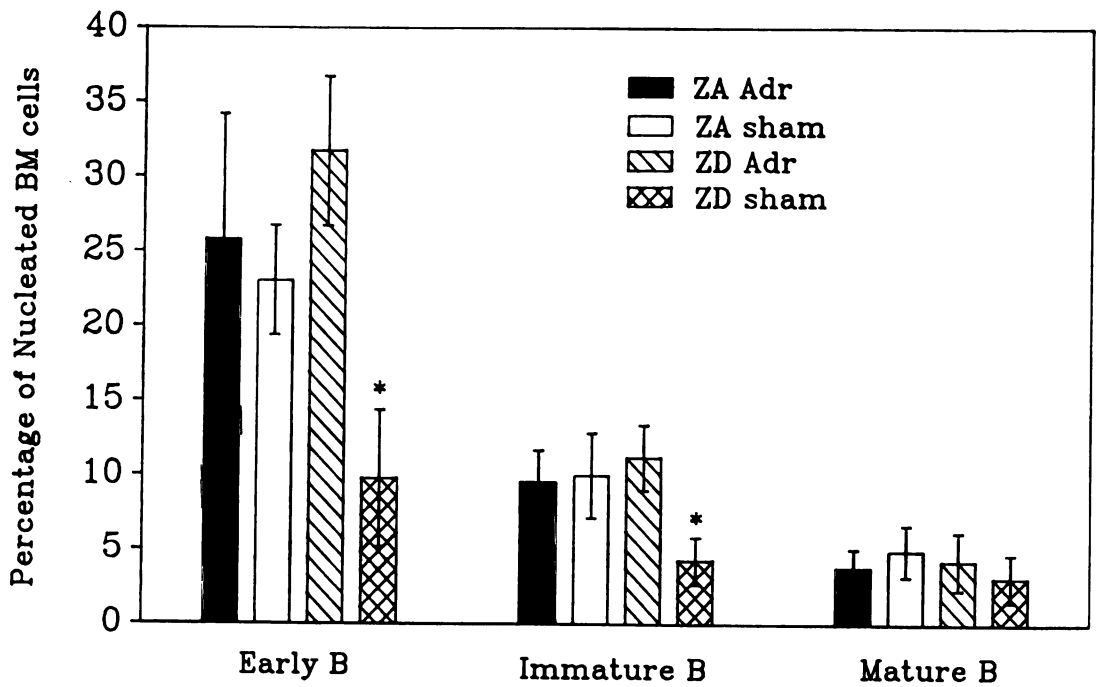

110
100
90
80
70
60
50
40
30
20
10
0
Plasma Zn (μg/dl)
**
*
ZA Adr
ZA sham
ZD Adr
ZD sham

Figure 4: Proportion of early developing ($B220^+Ig^-$), immature ($B220^+IgM^+IgD^-$) and mature ($B220^+IgM^+IgD^+$) B-cells in the marrow of mice that were sham operated and maintained on zinc deficient diet (ZD sham) or zinc adequate diet (ZA sham) or which were adrenalectomized and maintained on zinc deficient (ZD Adr) or zinc adequate diet (ZA Adr). Fluorescently labelled antibodies were used to quantitate the proportion of $B220^+$, IgM^+ and IgD^+ cells in the marrow of each dietary group as determined by flow cytometry where data from 10,000 cells were collected for each analysis. Data are expressed as the mean values of the percentage of all nucleated cells of the marrow for each population ± SD, and represent two separate studies. The number of mice per treatment group ranged from n = 6 to 8. * Denotes numbers significantly different from ZA sham and ZD Adr mice at $P < 0.05$, where separate comparisons were performed.


Percentage of Nucleated BM cells
40
35
30
25
20
15
10
5
0
ZA Adr
ZA sham
ZD Adr
ZD sham
*
*
Early B
Immature B
Mature B

CHAPTER FIVE

Evaluation of the Effects of Zinc Deficiency on T-cell Maturation in the Thymus of Young Adult A/J Mice: A Possible Role for Apoptosis in Zinc Deficiency

Abstract

The presence of thymic atrophy and lymphopenia and the disruption of B-lymphopoiesis in zinc deprived mice led to the investigation of two main questions in this study. The first question was to determine how suboptimal zinc intake affects phenotypic distribution of major thymic T-cell subsets including progenitor ($CD4^-CD8^-$), immature ($CD4^+CD8^+$), and mature ($CD4^+CD8^-/CD4^-CD8^+$) T-cells. The second question was to determine if apoptosis was the means for the elimination of susceptible lymphocytes in the course of dietary zinc deficiency. To investigate the first objective, young adult A/J mice were provided zinc adequate (ZA) or zinc deficient (ZD) diet for 27 days. This was followed by flow cytometric evaluation of the phenotypic distribution of T-lymphocyte subsets in the thymus of mice in zinc dietary study. The results from this investigation indicate that T-lymphocyte subsets are selectively affected by the deficiency. Phenotypic analysis of thymic T-lymphocytes revealed a significant resistance of double negative (DN) progenitor ($CD4^-CD8^-$) as well as single positive (SP) mature ($CD4^+CD8^-$/ $CD4^-CD8^+$) T-cells to the effects of ZD. By contrast, the proportion of double positive (DP) immature T-cells ($CD4^+CD8^+$) showed marginal decline in moderately affected (MZD) and greater depletion (15%) in severely affected mice by zinc deficiency (SZD). However, when the absolute number of this population was evaluated a substantial decrease (38%) in MZD mice and a significant reduction (86%) in SZD group were noted. Furthermore, moderate levels of zinc deficiency (MZD) caused a 41% depletion in thymic cellularity, with substantial depletion (83%) in SZD mice. The suppression in T-cell numbers

was directly correlated to a 36 and 67% involution of the thymus in MZD and SZD groups, respectively.

Determination of apoptosis in zinc deficiency, the second objective, was investigated both *in vivo* and *in vitro*. However, due to rapid clearance of apoptotic cells by phagocytic cells *in vivo*, detection of apoptosis after 27 days of diet study was hardly possible. Thus, the *in vivo* approach utilized a short incubation (6 hrs) of thymic T-cells from mice in a 27-day dietary zinc study in regular culture media (RPMI-1640), to reveal apoptosis in cells that had received the death signal *in vivo*, but had escaped detection by phagocytic cells. This approach was able to detect a 23% level of apoptosis in immature T-cells of SZD mice, whereas MZD mice showed almost the same level of apoptosis in this population (17%) as did control ZA fed mice (16%). This result indicated that perhaps most of the apoptotic thymocytes had already been cleared *in vivo* by phagocytic cells and only a very small portion of apoptotic cells had the chance to escape *in vivo* phagocytosis and to be captured by this system. To bypass this problem, simulation of ZD - though for a short time- in an *in vitro* culture system was investigated. This approach utilized a short incubation (8 hrs) of regular mouse thymic T-cells in culture media (RPMI-1640) to which known amounts of zinc (Zn) and corticosterone (CS) at the estimated levels in ZA and SZD mice, were added. This system detected a substantial percentage of apoptotic T-cells particularly among immature T-cells (2.8 fold) in the SZD simulated culture media compared to the control ZA simulated culture condition. Taken together, the data presented herein indicate the selective sensitivity of thymic T-lineage subsets to the effects of zinc deficiency and verifies the occurrence of apoptosis in the thymus of SZD mice.

Introduction

Previous investigations on the evaluation of the effects of suboptimal zinc intake on the immune system has strongly documented the immune impairment, manifested as thymic atrophy, lymphopenia and impaired antibody and cell mediated immunity (Fraker *et al.*, 1986; 1987; Keen and Gershwin, 1990; Zeng *et al.*, 1991; King *et al.*, 1995). In addition, the results from previous chapters (2, 3, and 4) indicated that ZD resulted in stage-specific alteration in BM B-lymphocytes which were otherwise protected in adrenalectomized mice. However, the status of phenotypic distribution of developing T-cells in the thymus of ZD mice, and the mechanism by which thymocytes were eliminated remained unclear. Thus, the objectives of the study presented herein were two fold. The primary objective was to investigate the relative sensitivity or resistance of thymic T-lineage cells (progenitor, immature, and mature T-cells) to the effect of ZD. It was of interest to see whether the results would show a pattern of alterations similar to that observed in BM B-lymphopoiesis in ZD mice. In this regard, thymocytes prepared from the three dietary groups (ZA, MZD, SZD) were phenotyped with monoclonal antibodies against T-cell maturation markers (CD4 and CD8) followed by flow cytometric analysis. This is the first detailed examination of cells of the T-lineage in the thymus of zinc dietary mice.

The second objective of this study was to investigate whether apoptosis played a role in the elimination of susceptible lymphocytes in zinc deficiency. One of the earliest investigations which linked ZD to apoptosis, was reported by Elmes (1977). He observed significant increase in the number of apoptotic bodies in the mucosa of small intestine of ZD

rats compared to ZA fed rats, suggesting the induction of apoptosis via deficiency in zinc. Martin and colleagues (1991) examined the survival of three cell lines of human lymphoid (Molt-3 and Raji) or myeloid (HL-60) origin *in vitro* under zinc sufficient or zinc deficient conditions. Zinc deficiency resulted in diminished proliferative capacity and viability of all the cell types. Cell death occurred mainly via apoptosis in the HL-60 and Raji cells and via necrosis in the Molt-3 cells. These studies suggested that ZD can induce apoptosis both *in vivo* and *in vitro*. A more recent study by Treves *et al.*, (1994) suggested a role for zinc as an intracellular regulator of apoptosis, since the chelation of zinc resulted in induction of DNA fragmentation into 200 bp in peripheral blood lymphocytes.

Parallel to the above studies, GC hormones are also thought to induce thymic atrophy *in vivo* through the enhancement of apoptosis (Munck and Grabtree, 1981; Compton *et al.*, 1987). It has been long recognized that mouse thymocytes undergo apoptosis in response to a variety of apoptotic cues including GCs (Wyllie, 1980; Morris *et al.*, 1984; Telford *et al.*, 1991; Nieto *et al.*, 1992; Cohen, 1992). These findings prompted the question of whether the sensitivity of immature thymocytes to the effects of ZD might be relevant to the *in vivo* and *in vitro* elimination of thymocytes via GC-induced apoptosis. Thymocytes have been extensively used over the past decade as an easy and reliable model for the study and characterization of GC-induced apoptosis (Wyllie, 1980; Cohen, 1992; Sun *et al.*, 1992; Gruber *et al.*, 1994; Korsmeyer, 1995). The homogeneity of the thymus (populates >90% thymocytes) not only makes it easier to assess apoptosis but also brings the opportunity to determine the effects of ZD on T-cell development.

Thus, the occurrence of apoptosis in ZD, as the second objective of this study, was investigated in the thymus both *in vivo* and *in vitro*. However, based on a small pilot study, we knew that the detection of apoptotic thymocytes after a period of four weeks diet study would not be successful, perhaps due to rapid clearance of apoptotic cells by macrophages *in vivo* (Wyllie *et al.*, 1980; Cohen 1991). Short incubation of thymocytes (6 hours), however, might allow those cells that had just received the death signal *in vivo*, to complete the apoptotic phase *in vitro* without detection by macrophages. To investigate, thymocytes prepared from different dietary groups were incubated in RPMI-1640 medium at 37°C for 6 hours, followed by immunophenotyping and DNA staining. Not to our surprise, this approach could only detect a small portion of the apoptotic lymphocytes occurring in a ZD mouse. Therefore, a second approach consisting of an *in vitro* culture system designed to simulate the *in vivo* CS and Zn levels of a ZA and a SZD mouse, was investigated. Although, the establishment of *in vivo* conditions of 28-day dietary mice in an 8 hrs-*in vitro* culture system is absolutely impossible, the *in vitro* system presented herein was an attempt to test the survivability of thymocytes in culture conditions where Zn and CS levels were analogous to the levels observed in ZA and SZD mice. Since addition of CS to the culture media at levels detected in zinc dietary mice (ZA and SZD) resulted in >70% cell death in thymocytes (preliminary data), the estimated free CS (unbound CS in plasma) levels which is believed to exert the biological activity of GCs (Faict *et al.*, 1985; Vermeulen , 1986), were utilized. In this system thymocytes prepared from regular mice were incubated for 8 hours in RPMI-1640 culture media supplemented with estimated levels of Zn (50 or 100 μg/dl) and free CS (2 or 6 μg/dl) found in ZA and SZD mice, respectively. Subsequent to incubation, thymocytes

were subjected to two color immunofluorescent phenotyping against major T-cell surface markers (CD4 and CD8) followed by DNA staining to detect apoptosis in distinct T-cell subsets.

The data presented in this study indicates the high susceptibility of immature thymocytes and the relative resistance of progenitor and mature T-cells to the effects of ZD. Furthermore, the high incidence of apoptosis in thymocytes, particularly in immature subpopulation, indicates the presence of apoptosis in ZD, and suggests that it plays a significant role in the elimination of vulnerable lymphocytes (lymphopenia) in the course of the deficiency.

Materials and Methods

Mice and Diet:

Six week old female A/J inbred mice (Jackson laboratory Bar Harbor, ME) weighing 17.1+ 0.7 g were used throughout the study. All mice were fed ad libitum a biotin fortified egg white based diet which contained either 30μg Zn/g diet (zinc adequate group; ZA) or ~0.5μg Zn/g diet (zinc deficient group; ZD). The composition of the diet has been described in Chapter 3. All dietary groups were maintained in stainless steel cages with mesh bottoms to reduce recycling of zinc for a period of 27 days. Feed jars and water bottles were washed in 4N HCl and the drinking water was acidified to reduce *Pseudomonas* infections. Food consumption was recorded daily and body weights were measured twice a week. At the end of the dietary study, the ZD group was subdivided into moderately affected mice by zinc deficiency (MZD) weighing an average of 78% of the ZA group with moderate signs of parakeratosis of the ears and tail or severely affected mice by zinc deficiency (SZD) weighing an average of 71% of the ZA group which exhibited more severe parakeratosis.

Blood Collection and Plasma Corticosterone Assay:

To prevent elevation of endogenous corticosterone and maintain it at the lowest levels during the diurnal cycle, mice were bled within 90 seconds of disturbing the cages between 8-9 in the morning. Blood collected from the subclavian arteries of anesthetized mice from all three dietary groups, was processed individually in acid washed heparinized microtubes. A spectrofluorometric method for measuring corticosterone in small volume of plasma (Garvy

et al., 1993a) was utilized. Briefly CS was extracted from 30 µl of plasma by addition of 600 µl dichloromethane (Aldrich, Milwaukee, WI). After vigorous shaking and centrifugation, the aqueous layer (top phase) was discarded. Then the solvent layer (bottom phase) was treated with 0.1N NaOH, mixed very well and centrifuged. The organic phase (bottom layer) was then used for the fluorescence development. A volume of 200 µl pre-fluorescence mixture consisting of three parts concentrated sulfuric acid (Aldrich, Milwaukee, WI) and one part absolute ethanol (200 proof) was added to the organic phase. To develop the fluorescence, samples were then vigorously shaken for 2 min., centrifuged and the solvent layer (top phase) was discarded by aspiration. After 30 min. incubation of the acid layer at room temperature, the fluorescence intensity was determined on a spectrofluorometer (Perkin Elmer, model 650-40) at 475 nm excitation and 525 nm emission. Standards containing 10-75 ng CS (Sigma, St. Louis, MO) were followed through the same procedure as plasma samples. Efficiency of the recovery of CS from plasma was determined by adding known concentration of standards to the plasma samples of known concentration and ranged between 85% to 95%.

Preparation of Thymocytes from Dietary Mice and Cell Culture for *In Vivo* Detection of Apoptosis:

Thymuses from mice in different dietary groups (6-8 mice per group) were removed, weighed, minced and passed individually through sterile 100 micron mesh stainless steel screen into sterile Hepes buffered Hank's balanced salt solution (HBSS) supplemented with 4% heat inactivated fetal bovine serum (FBS) (Hyclone,Logan,UT). Single cell suspensions

were washed twice, counted for total T-cell numbers (thymus cellularity) and tested for viability using trypan blue exclusion dye (>95% viability). The cells were then resuspended in RPMI-1640 supplemented with 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids, 50 μg/ml gentamicin and $5x10^{-5}$ M 2-mercaptoethanol (2-ME). Aliquots of cells (10^6 cells/ml/well) were plated in 24-well tissue culture plates (triplicates of each sample) and incubated for 6 hours at 37°C, 5% $C0_2$ atmosphere. Cultured thymocytes were then immunophenotyped and stained with DNA dye to evaluate the phenotypic distribution as well as apoptosis in distinct T-cell subsets.

Preparation of Thymocytes from Regular Mice for *In Vitro* Detection of Apoptosis:

Thymuses from young adult (6 to 10 weeks old) A/J female mice were removed and extruded through 100 micron stainless steel screen into modified HBSS supplemented with 2% FBS. Single cells were washed twice by centrifugation at 400 xg for 5 min., resuspended in HBSS/FBS, and counted using trypan blue-exclusion viability dye (>97% viable).

Absorption of GCs and Zn from Fetal Bovine Serum :

Since the core of the *in vitro* culture system was based upon the supplementation of the system with known amounts of CS and zinc, it was necessary to minimize the possible interference of the basal levels of GCs and zinc normally present in the FBS, with the *in vitro* assay described below. To do this, the FBS was treated with dextran coated charcoal which as reported removes GC from serum (Hayashi *et al.*, 1984). Subsequently, the charcoal

treated FBS was treated with Chelex-100 (Bio-Rad laboratories, Hercules, CA) using the batch method (Bio-Rad instructional manual) to remove zinc. Briefly, FBS (Hyclone,Logan,UT) was mixed with 1 mg/ml dextran (Sigma Chemical Co., St. Louis, MO) and 10 mg/ml Norit A activated charcoal (Matheson Cleman and Bell, Norwood,OH) and incubated for 30 min. in a 50°C waterbath subjected to frequent shaking. Dextran coated charcoal was removed from FBS by centrifugation at 4000 xg for 10 min. at 4°C and subsequent filtration through a 0.22 μm filter. These steps resulted in CS deficient FBS. To remove zinc from this serum, Chelex-100 was added to the FBS at 5 g/ml followed by gentle stirring on an electronic mixer for one hour. The resin was separated from FBS by filtration through a 0.22 μm filter and the FBS was stored at -20°C. The FBS obtained from these treatments (Zn^- CS^-) was periodically tested for Zn and CS levels both of which were below the detection limit of the assays (< 0.01 µg/dl). This serum was used to supplement the culture media to which known amounts of CS (2 or 6 µg/dl)(4-Pregnene-11B,21-diol-3,20-dione, Sigma Chemical Co., St. Louis, MO) and/or Zn (0, 50 or 100 µg/dl)(zinc sulfate heptahydrate, J.T.Baker, Phillipsburg, NJ) were added depending on the culture condition described below.

Culture Conditions for *In Vitro* Detection of Apoptosis:

RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 µg/ml of streptomycin, 100 IU/ml of penicillin, 50 µg/ml gentamicin, 1% non-essential amino acids, and $5x10^{-5}$ M 2-mercaptoethanol was used in this assay. This medium was then supplemented with one of the combinations illustrated in the chart below:

Culture Condition	FBS (CS^-Zn^-)	CS Added	Zn Added
1) Basal CS/basal Zn (BL CS/BL Zn)	20%	0.06 µM (2 µg/dl)	3.5 µM (100 µg/dl)
2) High CS/low Zn (HI CS/LO Zn)	20%	0.17 µM (6 µg/dl)	1.25 µM (50 µg/dl)
3) High CS/high Zn (HI CS/HI Zn)	20%	0.17 µM (6 µg/dl)	500 µM (14.3 mg/dl)
4) Basal CS/undetectable Zn (BL CS/UN Zn)	20%	0.06 µM (2 µg/dl)	no addition undetectable

Thymic T-lymphocytes prepared from regular mice were added at $2x10^6$ cells/ml/well to the various culture media in 24-well plates. The cultured cells were incubated for 8 hours at 37°C in 5% CO_2 atmosphere. Each culture condition was tested in quadruplicate.

Immunofluorescent Phenotyping and Staining of Apoptotic Cells:

After incubation of thymic T-cells from dietary mice in regular culture media (6 hrs), or thymic T-cells from regular mice in CS/Zn supplemented culture conditions (8 hrs), the percent recovery (100%) and the viability (>90%) of the cultured cells were examined. Aliquots of $1x10^6$ cells/ml were then resuspended in cold label buffer (HBSS, 2% FBS, 0.1% sodium azide), washed at least once, and immunophenotyped using antibodies to T-lineage surface markers. CD4 and CD8 surface markers on mouse T-cells were simultaneously labeled using 150 µl of phycoerythrin (PE) conjugated rat anti-mouse CD4 and fluorescein

isothiocyanate (FITC) conjugated rat anti-mouse CD8 monoclonal antibodies (Pharmingen, San Diego, CA). All labeling was performed at 4°C for 1/2 hour followed by two washes in cold label buffer. Furthermore, all the antibodies were used at their saturating concentration subsequent to titration.

To evaluate the presence of apoptosis in thymic T-lymphocytes, phenotyped samples were resuspended in one part (0.4 ml) cold phosphate buffer saline (PBS) supplemented with 50% FBS, and fixed by dropwise addition of three parts (1.2 ml) of cold ethanol followed by gentle mixing. Cells were kept overnight at 4°C, washed twice with cold label buffer to remove fixative and resuspended in DNA staining dye 4′,6-diamidino-2-phenylindole (DAPI) at 1 µg/ml. Stained samples were kept on ice prior to flow cytometric analysis.

Flow Cytometry:

Immunofluorescently labeled samples were analyzed on a Bacton Dickinson Vantage equipped with a consort 32 HP computer with LYSYS™ II and MultiPlus™ softwares. Fluorochrome excitation for one and two color analysis (PE, FITC and PE/FITC) was accomplished using an ILT model RCP-50 argon laser at 488 nm. Fluorochrome emission for FITC and PE was detected at 530±15 nm and 575±13 nm, respectively. For three color analysis of samples stained with DAPI, PE and FITC, simultaneous use of a Krypton laser exciting at 350 nm for DAPI and the argon laser was required. Negative controls included unstained thymocytes to define the negative population and detect autofluorescence. Cells labeled with isotype-matched non-specific antibodies were used to detect background fluorescence. Single and dual color controls were used to define the negative as well as the

positive population for each antibody and to set color compensation. Phenotypic distribution of T-cells as well as quantitation of apoptotic thymocytes were determined by initial gating through DAPI DNA width vs area fluorescence, excluding debris and doublets and including apoptotic cells. The gated population was then used for cytogram scatter phenotypic and cell cycle histogram analysis.

Statistical Analysis:

In order to identify any significant differences among dietary groups or different cell culture conditions, all the data were analyzed by the Kruskal-Wallis nonparametric test and the analysis of variance (ANOVA) for parametric data (Daniel, 1987). A Tukey's post-hoc comparisons was then applied where appropriate (Daniel, 1987). Differences were considered statistically significant at $P<0.05$. All the data are presented as the mean$\pm$SD of 6 to 8 mice for the dietary study, and quadruplicates of each culture condition for the *in vitro* culture samples.

Results

Effect of ZD on Body Weight, Thymus Weight, Thymus Cellularity, and Plasma CS Levels:

The effect of ZD on growth, thymus weight, thymic cellularity, and plasma CS levels of mice on a 27-day dietary study are presented in Table 1. On the last day of diet study, ZD mice were divided into MZD and SZD groups based on body weight and degree of parakeratosis. As shown in table 1, consumption of ZD diet for a period of about four weeks caused 22% to 29% drop in body weight in ZD group based on the degree of deficiency. The thymus weights in ZD mice declined significantly, with 36% to 67% depletion in the thymus weight of MZD and SZD groups, respectively, compared to that in ZA controls. Thymus cellularity was also affected by ZD. Mice in the MZD group lost 41% of their total thymocytes with more severe depletion of thymocytes (83%) observed in SZD group. The plasma CS levels exhibited significant elevation in ZD group, showing about 2.4 to 3.4 fold increase in MZD and SZD mice, respectively, compared to ZA mice. The presence of growth retardation, thymic involution as well as elevation of GC in this study resembles the previous diet studies (DePasquale-Jardieu and Fraker, 1980; King and Fraker, 1991; King *et al.*, 1995; Fraker *et al.*, 1995).

Effects of ZD on T-cell Maturation in the Thymus:

To evaluate the effects of ZD on phenotypic distribution of thymic T-cells, 6 hrs cultured thymocytes prepared from individual mice in each dietary group were

immunofluorescently labeled with monoclonal antibodies against major T-cell surface markers. The labeled cells were then fixed and stained with DAPI DNA binding dye for *in vivo* detection of apoptotic thymocytes which will be explained in the next section. The immunophenotyping of cells resulted in detection of four distinct thymic T-cell subsets, including progenitor ($CD4^-CD8^-$), immature ($CD4^+CD8^+$), and mature ($CD4^+CD8^-$)/($CD4^-CD8^+$) thymocytes. Figure 1 demonstrates the distribution of CD4/CD8 T-lymphocyte subsets in all dietary groups. As seen in figure 1A, the proportion of progenitor T-cells ($CD4^-CD8^-$) which are the earliest committed T-cells in the thymus was increased to +20% in MZD and +48% in SZD compared to the ZA controls. Likewise, the proportion of mature T-cells ($CD4^+CD8^-/CD4^-CD8^+$) exhibited +36% to +69% (Figure 1B) and +33% to +82% accumulation (Figure 1C), respectively, depending on the level of the deficiency. The immature T-cells ($CD4^+CD8^+$), however, showed moderate decline in MZD and a greater depletion (15%) in SZD group compared to ZA control mice (Figure 1D). This cell loss was more significant when the absolute number of $CD4^+CD8^+$ cells were examined for each dietary group (Figure 2). As Figure 2 demonstrates, the total number of immature T-cells in SZD mice dropped significantly to about 14% of ZA control group. Likewise, the immature population in MZD mice declined to a lesser degree, being 62% of that in ZA mice. When the absolute number of progenitor and mature T-cell compartments were evaluated, a moderate decline in each population was noted (data not shown).

***In Vivo* Verification of Apoptosis in ZD:**

The immunofluorescently labeled/DNA stained cultured thymocytes from mice in different dietary groups were evaluated for *in vivo* apoptosis. Two color phenotype-gated DAPI cell cycle analysis was applied to specifically investigate the degree of apoptosis in double positive thymocytes. As shown in Figure 3, a small percentage of $CD4^+CD8^+$ subset (16%) in ZA mice appeared in the apoptotic region of DNA histogram (Bottom panel). This may indicate the normal level of apoptotic death among self-reactive and non-functional T-cells in the thymus (Kisielow *et al.*, 1988; Murphy *et al.*, 1990). In the evaluation of ZD group, it was noted that while moderate levels of deficiency exhibited no significant change in the levels of apoptosis, severe deficiency in zinc resulted in 23% apoptosis in immature thymocytes (Figure 3). Progenitor and mature T-lymphocytes in ZD groups also showed some low degree of apoptosis which was not different from that of ZA controls (data not shown). These results indicated that perhaps most of the apoptotic cells had been eliminated by macrophages *in vivo*, leaving only a very small portion of apoptotic cells undetected by phagocytic cells. To overcome this problem, an *in vitro* culture system simulating ZD-though short- was designed. It was of interest to determine whether in a system where Zn and CS are added at the estimated levels observed in zinc dietary mice, and without the presence of macrophages, apoptosis could be detected.

Detection of Apoptosis *In Vitro*:

Thymic T-cells prepared from regular mice were incubated in different culture conditions (see the chart in materials and methods) for 8 hrs and were subsequently

immunophenotyped against CD4/CD8 T-cell surface markers with simultaneous DNA staining. As Figure 4 illustrates, total thymic T-cells in control ZA simulated culture condition (BL CS/BL Zn) demonstrated a low level of apoptosis (16%) similar to the *in vivo* level detected in ZA mice shown in Figure 3. By contrast, T-lymphocytes incubated in HI CS/LO Zn culture media (simulated culture condition of a SZD mouse) showed almost three fold increase in their apoptotic population (45% apoptosis) compared to the control (BL CS/BL Zn). Furthermore, among different thymic T-cell subpopulations, immature thymocytes showed the most sensitivity, comprising almost 80% of the total apoptotic events in mouse thymic T-cells (Figure 4). Moreover, addition of high concentrations of zinc (500 μM), which is known to inhibit apoptosis, to the SZD simulated culture media (HI CS/HI Zn) prior to incubation, suppressed apoptosis close to the background levels observed in control ZA simulated culture condition (Figure 4). It was also important to investigate how absence of zinc would affect T-cells survival. As it can be seen from Figure 4, short term incubation of cells in culture condition deficient in zinc (BL CS/UN Zn) showed significant apoptosis (~2 fold increase) in total T-cells from which 80% were immature thymocytes. Thus, this approach could demonstrate the significant increase in apoptosis in immature thymocytes treated in conditions in which CS and Zn levels closely resembled the *in vivo* levels in SZD mice.

Discussion

Earlier chapters (2 and 3) demonstrated that ZD exerts deleterious effects on B-cell genesis, mainly on early precursor and immature B-cells with no substantial effects on early progenitor or mature B-lymphocytes. Furthermore, it was shown (Chapter 4) that chronic elevation of GCs in ZD played a major role in thymic atrophy and alteration in B-cell development as elimination of this hormone via adrenalectomy protected thymic atrophy and lymphopenia and resulted in normal distribution of B-cell subcompartments in adrenalectomized ZD mice. The study presented herein extended our knowledge on lymphopoietic processes in ZD by providing the first detailed study on the status of T-cell development in zinc deficient mice. Furthermore it showed that apoptosis was a possible mechanism by which lymphocytes susceptible to suboptimal zinc intake and elevated GC were eliminated.

The availability of a panel of mAb directed to T-cell associated surface antigens of mice (CD4 and CD8) as well as DNA fluorochrome (DAPI) in conjunction with multiparameter FACS analysis allowed for the analysis of thymic T-cells carrying these antigens and their cell cycle status for detection of apoptotic population. The first objective of this study which was to evaluate the phenotypic distribution of thymic T-cells in mice in a zinc dietary study, clearly demonstrated that T-cell maturation was adversely affected by ZD similar to the observed alteration in B-lymphopoiesis (Chapters 2 and 3). The highest susceptibility among different thymic T-cell subpopulations was observed in DP immature ($CD4^+CD8^+$) thymocytes, in particular in the SZD group. This is analogous to the alteration in B-cell development where precursor B-cells exhibited the most sensitivity and losses in zinc

deprived mice (Chapter 3). Interestingly, both of these populations are heavily involved in Ig or TCR gene rearrangements which require many enzymatic and molecular activities most of which are Zn dependent (Vallee and Auld 1990; 1993; Coleman, 1992; Li *et al.*, 1993; Osmond *et al.*, 1994; Godfrey *et al.*, 1994). Sensitivity of the immature population was even more magnified when their absolute number was evaluated. This analysis demonstrated that as the level of ZD intensified it created greater losses in immature thymocyte population where in SZD almost 85% of the DP population were lost. By contrast, the proportion of progenitor and mature T-cells showed significant accumulation, specifically in SZD mice, suggesting the less sensitivity or to some degree the resistance of these populations to zinc deprivation. As was shown in earlier Chapters (2,3) progenitor and mature IgD^+ B-cells also demonstrated more resistance to the effects of ZD. These are the populations that their antigen receptor genes (Ig/TCR) are either in a germline configuration or have been successfully rearranged (Hardy *et al.*, 1991; Li *et al.*, 1993; Godfrey *et al.*, 1994; Pawlowski and Staterz, 1994), thereby, are not actively involved in enzymatic and molecular activities. These cells might be, thus, less susceptible to nutritional deficiency and subsequent cell loss.

The second objective of this study was to investigate the presence of apoptosis in ZD mice as a mean of elimination of lymphocytes susceptible to zinc deprivation. As was described earlier in this chapter, this investigation was examined via two approaches. In the first approach (*in vivo*), in which thymic T-cells from dietary mice were shortly incubated in regular media, moderate levels of apoptosis, specifically in immature thymocytes of SZD mice (23%) was noted. MZD mice, however, did not show a detectable change in their apoptotic T-cells compared to the control ZA mice. With regard to the significant depletion

in immature thymocytes of ZD mice, it was clear that this approach was only capturing a small portion of apoptotic cells that had survived the *in vivo* elimination by macrophages. Thus apparently the rapid clearance of apoptotic cells by macrophages *in vivo* was still a barrier in the detection of actual apoptotic events in immature thymocytes during ZD. It is well documented that apoptotic cells are rapidly recognized and cleared by macrophages through the expression of specific surface changes (eg., phosphatidyl serine expression) (Fadok *et al.*, 1992a; 1992b; 1993) before their membrane integrity is lost (Ren *et al.*, 1995).

To overcome this problem the second approach including the *in vitro* culture system was investigated. As shown in Figure 4, short time incubation of normal T-lymphocytes in media supplemented with basal levels of Zn and CS was accompanied with a low level (16%) of apoptotic T-cells in this culture condition. This level reflects the normal ongoing apoptotic events in thymocytes that will not survive during thymic education (positive and negative selections) (Hedrick and Eidelman, 1993; Tough and Sprent, 1994). However, as expected, a significant number of T-cells, in particular DP population, died apoptotically when Zn and CS in culture were adjusted to the levels of a SZD mouse. In fact, an early study by Elmes described the presence of apoptotic bodies in the small intestine of severely deficient rat (Elmes, 1977). This phenomenon was, however, explained to be due to decreased DNA synthesis. Furthermore, addition of high levels of Zn (500 μM) to the system suppressed apoptosis to the background levels observed in the control culture condition. This is in agreement with the literature in which zinc at high levels (500-1000 μM) has been shown to act as an apoptotic inhibitor of GC-induced apoptosis (Cohen and Duke, 1984). It has been suggested that the inhibitory action of zinc at high levels on GC-induced apoptosis might

occur at any steps during GC binding to its cytoplasmic GR, GR transformation or translocation in target cells (Fraker and Telford, 1995). When the deficiency of Zn by itself in the presence of basal levels of CS was examined (BL CS/UN Zn), a significant increase in apoptosis, specifically in DP population was observed. Similarly, culture media deprived of zinc also resulted in apoptotic death in certain lymphoid and myeloid cell lines, indicating the induction of apoptosis by zinc deprivation (Martin *et al.*, 1991). Thus it appears that zinc at suboptimal levels can by itself eliminate susceptible cells by triggering apoptosis (Telford and Fraker, 1995).

The pattern of alteration in T-cell maturation and the detection of apoptosis both *in vivo* and *in vitro* in immature thymocytes, which are the core of sensitivity in the thymus of ZD mice, are all indications of a synergistic actions of zinc and GC in down sizing of the immune system. As mentioned in earlier chapters, the crucial role of zinc for cell growth, development, and differentiation as well as an essential component of more than 200 enzymes has been well documented (Vallee and Auld, 1993; Walsh *et al.*, 1994; Cousins, 1996). Considering these facts, one would expect that cells that are more metabolically or enzymatically active would be in more need for this nutritional element. Thus in situations where zinc is in suboptimal levels, cells such as immature thymocytes that are actively involved in TCR gene rearrangement and require zinc for their zinc-dependent biochemical activities would be sensitive to this deficiency and are programmed to die. On the contrary, cells of progenitor or mature T-cells that are either prior to gene rearrangement (TCR) or have successfully rearranged their gene tolerate this deficiency and are less prone to die.

In addition, chronic elevation of GCs in later stages of ZD and its induction of apoptosis specially in immature lymphocytes is another important phenomenon that must be considered in apoptotic depletion of sensitive lymphocytes in zinc deprivation. GC-induced apoptosis of lymphoid cells, in particular the immature lymphocytes of the thymus gland, is perhaps the most widely studied model of programmed cell death (Compton and Cidlowski, 1992; Cohen,1992). Furthermore, *in vitro* exposure of lymphocytes to low levels of synthetic or naturally occurring GCs resulted in accumulation of immature T and early B cells in apoptotic region of the cell cycle (Telford *et al.*, 1991; Garvy *et al.*, 1991; 1993a; 1993b; Voetberg *et al.*, 1994). These observations are strong indications of the suppressive effects of GCs on immature lymphocytes via induction of apoptosis.

Another consideration in observed alteration in T-cell development and its correlation to the apoptotic elimination of immature thymocytes is the expression of protooncogenes such as Bcl-2 and its related gene Bcl-x oncogene. The protooncogene Bcl-2 (apoptosis-suppressing gene) was the first gene studied in the context of program cell death (PCD) regulation (Reed, 1994; Korsmeyer, 1995). Over expression of Bcl-2 in DP thymocytes of transgenic mice enhances the resistance of these cells to γ-radiation and GC-induced PCD (Sentman *et al.*, 1991; Strasser *et al.*, 1994). Elimination of Bcl-2 by gene targeting in mice results in progressive apoptosis of B and T lymphocytes beginning at 3 to 4 weeks of age (Nakayama *et al.*, 1993; Veis *et al.*, 1993). Based on these experiments it appears that Bcl-2 regulates the survival of lymphocytes in response to a variety of stimuli that induce PCD.

As in the case of B-cell development explained in Chapter 3, the pattern of Bcl-2 expression in T-lymphocytes closely resembles the stage specific sensitivity of T-cells to

ZD/GC-induced apoptosis. Murine Bcl-2 is expressed in the developmentally early $CD4^-CD8^-$ progenitor T-cells, but diminishes as T-cell differentiation progresses into the $CD4^+$ $CD8^+$ DP stage such that little or no Bcl-2 is detected in immature thymocytes (Veis *et al.*, 1993) - the stage at which self/non-self screening and negative selection occurs. This is followed by upregulation of Bcl-2 in mature T- cells. Making the T-cell development scenario potentially more complex is the recent observation that expression of apoptotic-promoting Bcl-x_s peaks during the immature stage (Boise *et al.*, 1993), and it diminishes as cells progress to $CD4^+CD8^-$/$CD4^-CD8^+$ mature T-cells. The stage specific upregulation or downregulation of Bcl-2 and its related gene Bcl-x_s in thymocytes overlaps the stage specific resistance or susceptibility of thymocytes to the effects of ZD. Thus it could be suggested that in addition to deficiency of zinc and chronic elevation of CS as the main core underlying apoptotic elimination of immature thymocytes in ZD, the presence of other factors such as expression or absence of some oncogenes in the target cells could also contribute to the overall fate of the cells.

Collectively, it may be possible to suggest that at early stages of ZD prior to the elevation of GCs there might be some degree of apoptotic death among cells in need for this nutrient, largely due to deficiency in zinc. However, as ZD progresses, specifically in severe zinc deprivation, it activates stress axis and subsequent release of GC hormones that remains chronically elevated through the later stages of deficiency (DePasquale-Jardieu and Fraker, 1979; 1980; Fraker *et al.*, 1995). At this point GCs will possibly take over the situation by triggering cells that are susceptible to ZD (in this case immature thymocytes) to die apoptotically. This would limit the pool of zinc mostly available to those tissues that their

function is vital to the survival of the system. In fact high levels of GC hormones has been shown to redistribute zinc in the body (demonstrated in Chapter 3) and possibly increases zinc uptake by cells that are in need for this essential nutrient (Henkin, 1974). Thus,it appears that nature has set a series of events to protect vital tissues from adverse effects of this nutritional deficiency and insure the survival by downsizing the immune system via increased GC release and subsequent induction of apoptosis in susceptible cell populations.

Table 1: Body and thymus weights, thymus cellularity and plasma CS levels of mice after 27 days on zinc dietary study[a].

Dietary Groups	ZA	MZD	SZD
Initial body weight[b] (g)	17.2 ± 0.7	17.0 ± 0.5	17.0 ± 0.5
Final body weight[c] (g)	20.3 ± 1.4	15.8 ± 0.3*	14.4 ± 0.6*
Thymus weight (mg)	25.1 ± 1.1	16.2 ± 0.9*	8.4 ± 1.9*
Thymus cellularity ($x10^7$)	5.1 ± 1.7	3.0 ± 1.1*	0.85 ± 0.5*
Plasma CS (μg/dl)	17.6 ± 5.0	41.4 ±5.83*	59.8 ± 2.4*

a = Data represent two separate diet studies.

b = Mean ± SD of 18 mice on ZA and 24 mice on ZD diet on day 0 of diet study.

c = Mean ± SD of 6 to 7 mice in each dietary group assayed on day 27.

* = $p < 0.05$ or greater as compared to ZA mice.

Figure 1: Evaluation of the phenotypic distribution of thymic T-lymphocytes in ZA, MZD, and SZD groups after 27 days of diet. Incubated thymic T-lymphocytes from individual mice were immunofluorescently labeled with mAbs to CD4 and CD8 surface antigens with subsequent flow cytometric analysis. Proportions of **(A)** ($CD4^-CD8^-$) progenitor T-cells, **(B)** ($CD4^+CD8^-$) mature T-cells, **(C)** ($CD4^-$ $CD8^+$) mature T-cells, and **(D)** ($CD4^+CD8^+$) immature thymocytes in the thymuses of 27-day zinc dietary mice are presented. Data are expressed as mean $\pm$ SD of 6 to 8 mice in each dietary group, representing two separate diet studies. * indicates significantly different from ZA controls at $P < 0.05$.

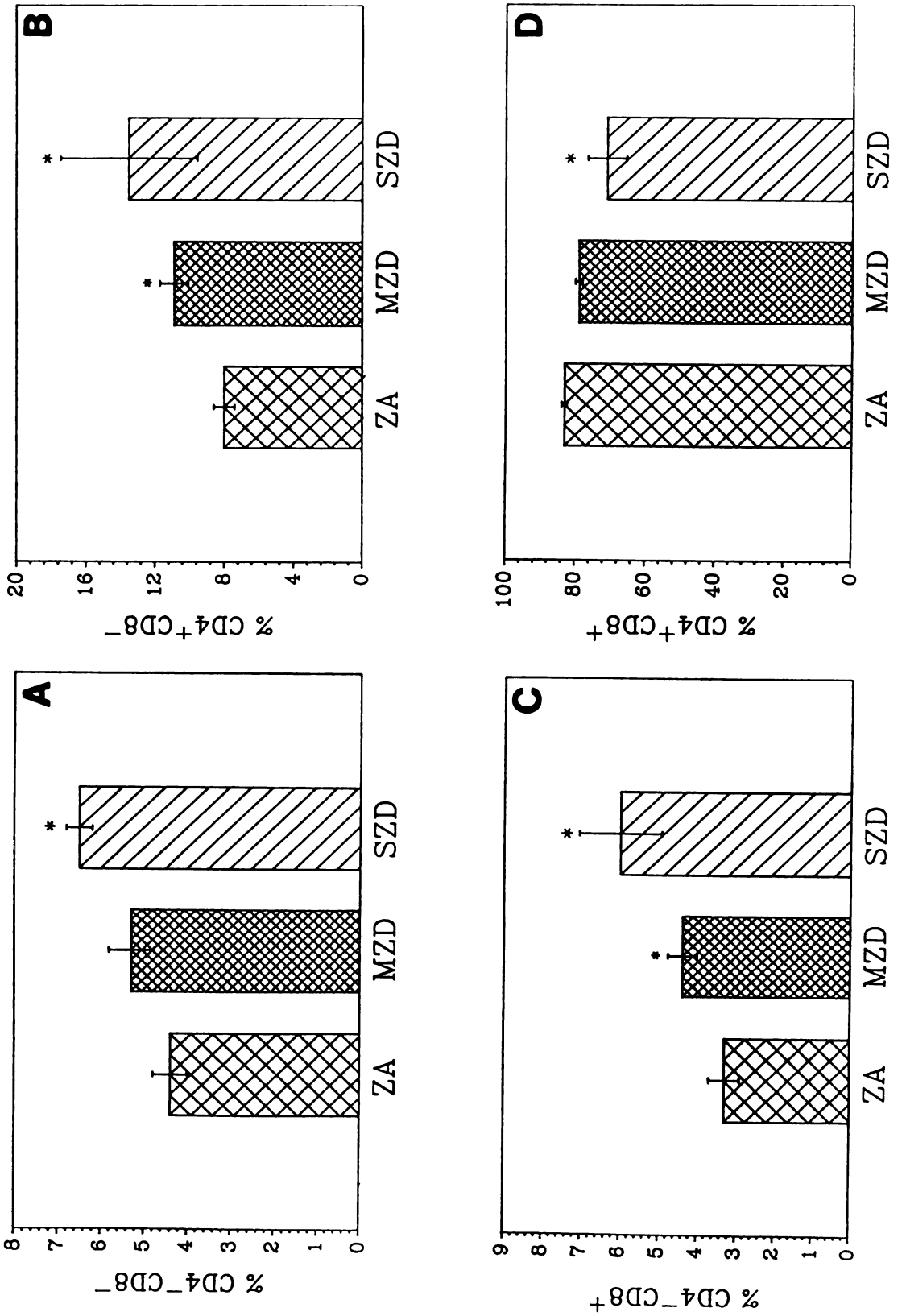

A
B
C
D
% CD4⁻CD8⁻
% CD4⁺CD8⁻
% CD4⁻CD8⁺
% CD4⁺CD8⁺
ZA
MZD
SZD
*

Figure 2: Absolute number (total numbers) of immature thymocytes in the thymus of ZA, MZD, and SZD mice. The total number of immature $CD4^+CD8^+$ T-cells in each dietary group was determined as: % $CD4^+CD8^+$ thymocytes in each mouse multiplied by the total number of T-cells in that corresponding mouse. Data are mean $\pm$ SD of 6 to 8 mice in each dietary groups and represent two separate zinc dietary study. * denotes significantly different at $P < 0.05$ as compared to ZA mice.

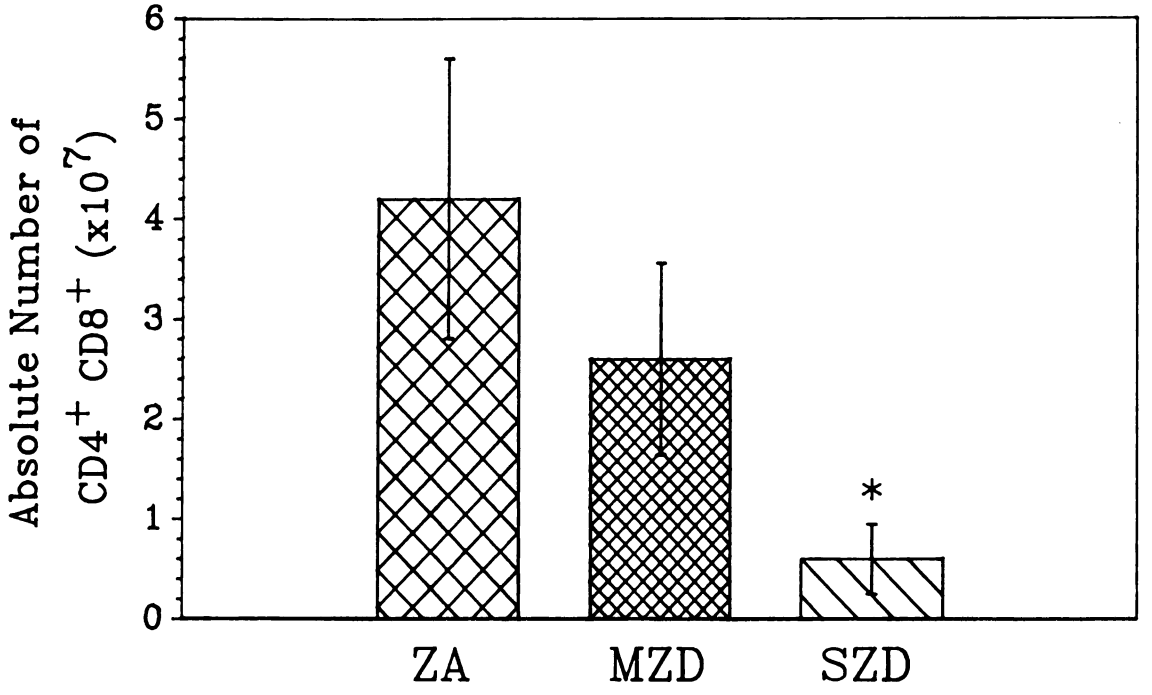

Absolute Number of
CD4+ CD8+ (x10^7)
6
5
4
3
2
1
0
*
ZA
MZD
SZD

Figure 3: Apoptosis in immature thymocytes of mice from ZA, MZD, and SZD groups at day 27. After 6 hrs incubation of thymic T-lymphocytes in RPMI-1640, cells were immunophenotyped and stained with DNA dye and subsequently the proportion of apoptotic thymocytes in each dietary group was determined. **Top panel** shows the proportion of apoptosis in immature DP ($CD4^+CD8^+$) thymocytes in all three dietary groups. **Bottom panels** represent the cell cycle histogram analysis of the data in the top panel with emphasis on the accumulation of apoptotic thymocytes in the hypodiploid A_o (apoptoic) region of the cell cycle of ZA, MZD and SZD groups. Data are expressed as the mean $\pm$ SD of 6 to 8 mice in each dietary groups and are representative of two separate diet studies.

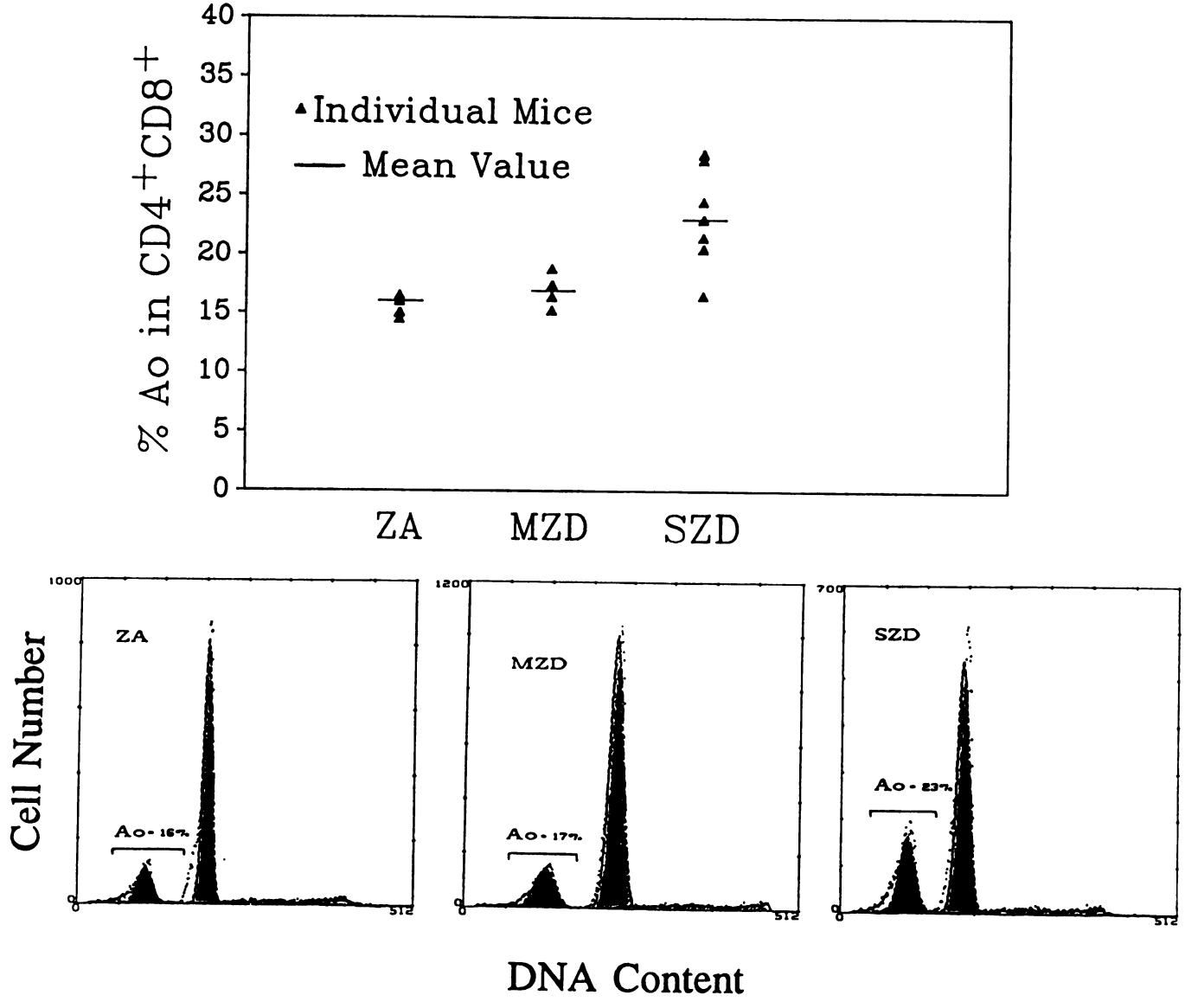
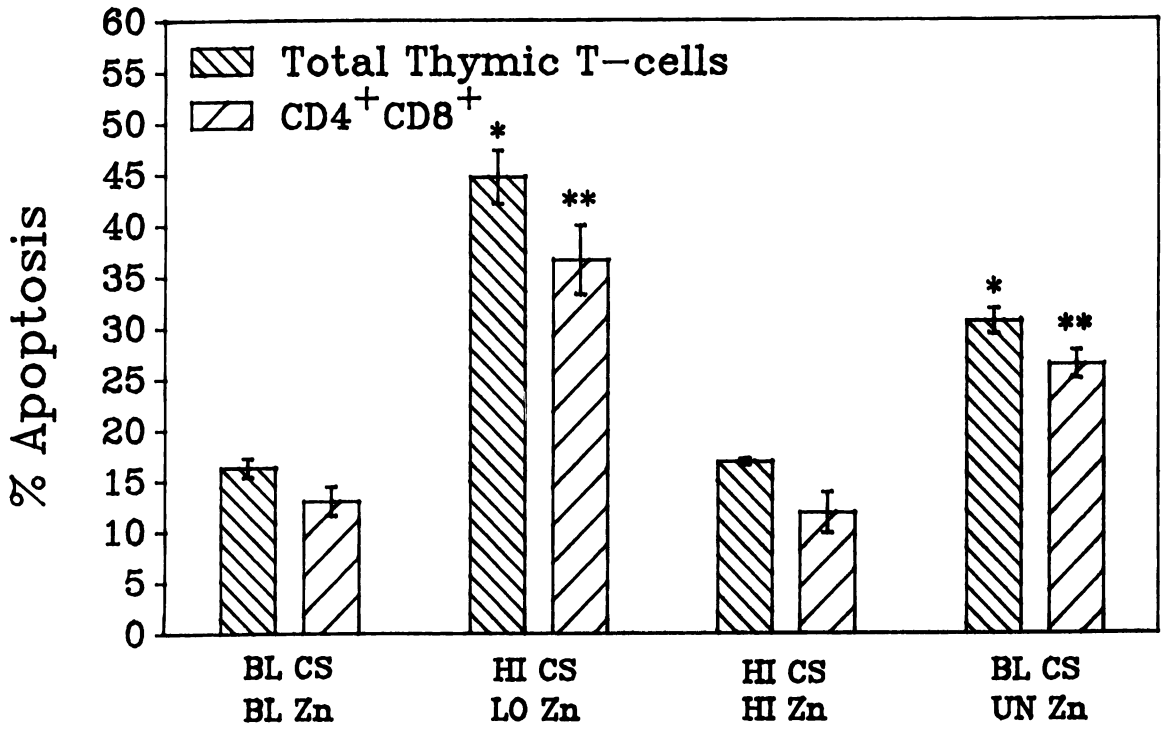

40
35
30
25
20
15
10
5
0
% Ao in CD4+CD8+
Individual Mice
Mean Value
ZA
MZD
SZD
Cell Number
ZA
Ao - 16%
MZD
Ao - 17%
SZD
Ao - 23%
DNA Content

Figure 4: Apoptosis in thymic T-cells in *in vitro* culture system. Thymic T-lymphocytes from regular mice were incubated in RPMI-1640 culture media with 20% CS^- Zn^- FBS supplemented with various CS and Zn concentrations for the period of 8 hrs. Cells were then immunophenotyped and stained with DNA dye and were subsequently analyzed by flow cytometry as described in the text. Basal CS/basal Zn (BL CS/BL Zn) (control) represents CS at 2 μg/dl and Zn at 100 μg/dl; high CS/Low Zn (HI CS/LO Zn) represents CS at 6 μg/dl and Zn at 50 μg/dl; high CS/high Zn (HI CS/HI Zn) indicates CS at 6 μg/dl and Zn at 500 μM (14.3 mg/dl); and basal CS/undetectable Zn (BL CS/UN Zn) indicates CS at 2 μg/dl with no addition of zinc to the culture. Data are expressed as mean ± SD of quadruplicates of each treatment and representative of three separate experiments. * indicates significantly different ($P < 0.05$) from total thymocytes in control group (BL CS/BL Zn). ** Denote significantly different ($P < 0.05$) compared to $CD4^+CD8^+$ thymocytes in the control group.


% Apoptosis
60
55
50
45
40
35
30
25
20
15
10
5
0
Total Thymic T−cells
CD4+CD8+
*
**
*
**
BL CS
BL Zn
HI CS
LO Zn
HI CS
HI Zn
BL CS
UN Zn

Summary and Suggestions

The world wide prevalence of zinc deficiency with deleterious effects on the immune system demanded investigation on the specific effects of this deficiency on lymphocyte subpopulations. The occurrence of lymphopenia in zinc deficiency prompted the question of whether the deficiencies in zinc interfered with lymphopoietic processes. Furthermore, it was important to determine if elevation of glucocorticoids associated with ZD was playing a role in the alteration of lymphopoiesis, as immunosuppressive effects of GCs are well established. Finally, the possibility of apoptotic death as a mean for elimination of vulnerable lymphocytes in ZD was investigated.

The results presented in this dissertation clearly demonstrate for the first time that B-cell development is adversely affected by ZD. Examination of the marrow of ZD mice revealed that small nucleated BM cells representing the lymphocyte population in the marrow were significantly diminished, whereas a significant accumulation in the myeloid lineage was noted. Precursor B-cells were the most severely affected population, followed by a significant depression in the proportion of immature B-cells in the BM of zinc deprived mice. By contrast, progenitor B-cells which are the earliest identifiable committed cells in the B-lineage exhibited substantial resistance to the deficiency. Likewise, the mature B-cell subpopulation demonstrated much less sensitivity, being less affected by the deficiency. In spite of significant depletion in some B-cell subcompartments, the marrow cellularity was unaffected, showing the analogous number of BM nucleated cells in all dietary groups. This finding

indicated the resistance of other lineages (eg., myeloid) in the marrow of ZD mice as was identified earlier.

Evaluation of the effects of ZD on T-cell maturation in the thymus revealed alteration in T-lymphocyte subsets in a pattern analogous to the B-lymphocyte subpopulations in the marrow. That is, the progenitor and mature T-cells showed substantial resistance to the effects of ZD, whereas immature thymocytes were depleted significantly. Together, these observations clearly indicated a selective and stage specific alterations in B-lymphopoiesis as well as in T-cell maturation in the course of ZD.

In investigation of the zinc content of several lymphoid and non-lymphoid tissues, a significant depletion in the bone zinc with no change in the zinc concentration in the BM and liver cells was noted. This pattern suggested a possible mobilization of zinc from bone to other tissues such as BM and liver as has been also indicated by other investigators. However, dietary studies terminated at different time points in which the zinc content of larger number of tissues are evaluated would better identify the kinetics of zinc depletion and the possibility of zinc redistribution in the course of the deficiency. Furthermore, the assessment of the zinc content of sorted populations (eg., $B220^{+}Ig^{-}$, $B220^{+}Ig^{+}$, $CD4^{+}CD8^{+}$) along with the determination of their phenotypic distribution at various time points in the course of dietary study would be able to better correlate the intracellular zinc levels to the survivability of each population. However, in order to specifically identify the role of suboptimal zinc levels by itself, the use of adrenalectomized mice in which there is no interference of GC hormones is suggested.

Alteration in lymphopoiesis along with the unchanged zinc levels in the marrow of ZD mice also suggest the contribution of other factors secondary to ZD. As it is documented, progression of ZD activates the stress axis and results in chronic release of glucocorticoids which remain elevated throughout the deficiency. Due to the immunosuppressive effects of GCs on lymphocytes, particularly on immature populations, their role in the alteration of lymphopoiesis during ZD was speculated. In this regard, removal of CS via adrenalectomy clearly showed that ZD adrenalectomized mice exhibited complete thymic protection and normal phenotypic distribution in early developing, immature and mature B-cell subpopulations compared to ZD sham control mice. This data identified a significant role for CS in the suppression of lymphopoiesis in zinc deprived mice. Furthermore, the accumulation of glucocorticoid resistance cells of the myeloid lineage in the marrow of ZD mice emphasized the role of GC as one possible mechanism in the suppression of vulnerable lymphocytes. Nevertheless, other possibilities such as alteration in the rate of lymphopoiesis and cell cycling can not be ruled out. The necessity of zinc for cell division and proliferation along with the presence of elevated GC during ZD, increases the possibility of reduced rates of lymphopoiesis and cycling in early developing B-lymphocytes. Using the FACS methodology developed in this laboratory along with metaphase arrest techniques (eg., use of vincristine to block cells at G_2/M phase) in a time point diet study would identify the contribution of these parameters to the observed lymphopenia in zinc deprived mice. Furthermore, interrelating these parameters to the plasma zinc and CS levels, as well as zinc content of lymphoid tissues would provide a more detailed picture of how gradual changes

in the zinc and CS levels in the course of ZD would play a role in lymphopoietic processes and determine the eventual fate of lymphocytes.

Over expression or down regulation of some of the oncogenes particularly Bcl-2 and its family members (eg., Bcl-x, A1, Bax, Bak, etc.) at different stages of B and T-cell development could also determine cell survival or cell death to maintain the potentially functional cells and also homeostasis of the system . As a clear example, Bcl-2, an anti-apoptotic oncogene has been shown to be expressed in progenitor and mature B-cells and down regulated in precursor and immature B-cells. The pattern of on and off expression of this oncogene in these lymphocytes overlaps with the resistance or the sensitivity of different B-cell subpopulations to the effects of ZD, namely GCs. Thus, the expression or down regulation of oncogenes could render a cell either sensitive or resistance to deficiencies in zinc or its secondary outcomes (eg., GC hormones).

Along with these factors, there are other possibilities that might contribute to the selective sensitivity of lymphocytes in zinc deprivation, one of which is the BM microenvironment in which lymphopoiesis occurs. Stromal cells, as one of the key components of this microenvironment, provide a substantial support for lymphohemopoietic processes. This is achieved via production of different cytokines and expression of various cell adhesion molecules that are involved in growth, maturation and differentiation of different cell lineages. It is possible that in the course of zinc deficiency the organization of this microenvironment is altered in such a way that cells that are greatly dependent on cell contacts or specific cytokines for their survival would somehow lose these supportive elements and, therefore, become eliminated in the course of ZD. Our insufficient knowledge

about the status of the microenvironment of the BM or thymus during zinc deprivation, demands extensive investigations which will likely reveal some explanations for the observed immunological defects due to this nutritional deficiency.

The results from the *in vivo* verification of apoptosis in ZD mice showed a moderate level of apoptosis in immature thymocytes detected by flow cytometric cell cycle analysis. However, due to rapid elimination of apoptotic cells by macrophages *in vivo*, it was speculated that the data was not a true representative of apoptotic events occurring *in vivo*. Therefore, an *in vitro* culture system with supplementation of different levels of zinc and CS analogous to their levels in dietary zinc mice was established. Data from these experiments clearly showed that in culture conditions where zinc was as low and CS was as high as their levels in ZD mice, a significant accumulation in apoptotic thymocytes, particularly, in immature thymocytes were evident. Furthermore, addition of high amounts of zinc to this culture condition substantially inhibited apoptosis, maintaining it at basal levels detected in the control media. These data suggested that apoptosis apparently occurs in ZD mice and might be a mechanism for the elimination of susceptible lymphocytes in ZD, thus creating lymphopenia. Nevertheless, more thorough investigations employing time point diet studies would greatly enhance the possibility of capturing apoptotic lymphocytes *in vivo* before their rapid clearance by macrophages.

In conclusion, the work described here has shed considerable light on the status of lymphopoietic processes in zinc deprived mice and has provided some insights into the possible roles of glucocorticoids and apoptosis in the observed lymphopenia. Although, future studies examining the role of the affomentioned factors in the impaired immunity in

zinc deficiency are highly required, the importance of zinc in the integrity of immune system and the its deleterious effects on immune components were substantially provided by this project. More importantly, this work has laid a foundation for further investigations which are likely to provide considerable insight into the treatment of human subjects, particularly children, that are either malnourished due to improper diet or are suffering from different diseases that would lead them to malnourished state.

LITERATURE CITED

Abbasi, A.A., Prasad, A.S., Rabbani, P., *et al.* (1980) Experimental zinc deficiency in man: effect of testicular function. J. Lab. Clin. Med. 96, 544-550.

Abdulwajid, A.W., and Wu. F.Y-H. (1986) Chemical modification of *Escherichia coli* RNA polymerase by diethyl pyrocarbonate: evidence of histidine requirement for enzyme activity and intrinsic zinc binding. Biochemistry 25, 8167-8172.

Abe, S., Matsumi, M., Tsukioki, M., *et al.* (1987) Metallothionein and zinc metabolism in endotoxin shock rats. Exs. 52, 587-594.

Adcock, I.M., Lane, S.J., Brown, C.R. *et al.* (1995) Differences in binding of glucocorticoid receptor to DNA in steroid-resistant asthma. J. Immunol. 154, 3500-3505.

Aggett, P.J., Delves, H.T., Thorn, J.H., *et al.* (1981) The therapeutic effect of amphotericin in *Actodermatitis enteropathica*. Eur. J. Pediatr. 137, 23-25.

Aggett, P.J., and Comerford, J.G. (1995) Zinc and human health. Nutr. Rev. 53, 516-522.

Aggett, P.J., and Harries, J.R. (1979) The current status of zinc in health and disease states. Arch. Dis. Child. 54, 909-917.

Aggett, P.J. (1989) Severe zinc deficiency. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 259-279.

Allman, D.M., Ferguson, S.E., and Cancro, M.P. (1992) Peripheral B cell maturation. I. Immature peripheral B cells in adults are heat-stable antigen^{high} and exhibit unique signaling characteristics. J. Immunol. 149, 2533-2540.

Allman, D.M., Ferguson, S.E., Lentz, V.M., and Cancro, M.P. (1993) Peripheral B cell maturation. II. Heat-stable antigen^{high} splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. J. Immunol. 151, 4431-4444.

Alroy, I., and Freedman, L.P. (1992) DNA binding analysis of glucocorticoid receptor specificity mutants. Nucl. Acids Res. 20, 1045-1052.

American Institute of Nutrition (1993) AIN-93 purified diets for laboratory rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J. Nutr. 123, 1939.

Anderson, G., Jenkinson, E.J., Moore, N.C., and Owen, J.J.T. (1993) MHC class II$^+$ thymic epithelium and mesenchyme cells are both required for T-cell development in the thymus. Nature 362, 70-73.

Anderson, G., Owen, J.J.T., Moore, N.C., and Jenkinson, E.J. (1994) Thymic epithelial cells provide unique signals for positive selection of CD4$^+$CD8$^+$ thymocytes *in vitro*. J. Exp. Med. 179, 2027-2031.

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

Atherton, D.J., Muller, D.P.R., Aggett, P.J., and Harries, J.T. (1979) A defect in zinc uptake by jejunal biopsies in *Acrodermatitis enteropathica*. Clin. Sci. 56, 505-507.

Auld, D.S., Kawaguchi, H., Liningston, D.M., and Vallee, B.L. (1975) Zinc reverse transcriptase from mammalian RNA type C viruses. Biochem. Biophys. Res. Commun. 62, 296-302.

Auphan, N., DiDonato, J.A., Rosette, C., *et al.* (1995) Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of I-κB synthesis. Science 270, 286-290.

Bach, J.F., Dardenne, M., Pleau, J.M., and Bach, A.M. (1975) Isolation biochemical characteristics, and biological activity of a circulating thymic hormone in the mouse and in the human. Ann. N.Y. Acad. Sci. 249, 186-210.

Ballard, P.L., Ballard, R.A., Gonzales, I.K., *et al.* (1984) Corticosteroid binding by fetal rat and rabbit lung in organ culture. J. Steroid Biochem. 21, 117-126.

Ballard, P.L. (1979) Delivery and transport of glucocorticoids to target cells. In J.D. Baxter, and G.G. Rousseau (eds), *Glucocarticoid hormone receptors*, Springer-Verlag, Berlin and New York, pp 25-48.

Ballester, O.F., and Prasad, A.S. (1983) Energy, zinc deficiency, and decreased nucleoside phosphorylase activity in patients with sickle cell anemia. Ann. Intern. Med. 98, 180-182.

Banda, N.K., Bernier, J., Kwahara, D.K., *et al.* (1992) Crosslinking CD4 by human immunodeficiency virus gp 120 primes T cells for activation induced apoptosis. J. Exp. Med. 176, 1099-1106.

Barone, K.S., O'Brien, P.C.M., and Stevenson, J.R. (1993) Characterization and mechanisms of thymic atrophy in protein malnourished mice: role of corticosterone. Cell. Immunol. 148, 226-233.

Barry, M., Keeling, P. W. N., and Feely, J. (1990) Tissue zinc status and drug elimination in patients with chronic liver disease. Clin. Sci. 78, 547-549.

Bateman, A., Singh, A., Kral, T., and Solomon, S. (1989) The immune hypothalamic-pituitary-adrenal axis. Endocr. Rev. 10, 92-112.

Baum, M., and Quigley, R. (1991) Parental glucocorticoids stimulate neonatal juxtamedullary prominal convoluted tubule acidification. Am. J. Physiol. 261, F746-F752.

Baxter, J.D., and Tyrrell, J.B. (1987) The adrenal cortex. In P. Felig, J.D. Baxter, A.E. Broadus, and L.A.Frohman (eds), *Endocrinology and Metabolism, 2nd ed.*, McGraw-Hill, New York, pp 511-650.

Baxter, J.D., and Rousseau, C.G. (1979) Glucocorticoid hormone action: An overview. In J.D. Baxter, C.G. Rousseau (eds), *Glucocorticoid Hormone Action*, Springer-Verlag, Berlin, Heidelberg, and New York, pp 1-24.

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

Beaumont, K., and Fanestil, D.D. (1983) Characterization of rat brain aldosterone receptors reveals high affinity for corticosterone. Endocrinology 113, 2043-2051.

Becker, D.J. (1983) The endocrine responses to protein calorie malnutrition. Ann. Rev. Nutr. 3, 187-212.

Berczi, I. (1994) Hormonal interactions between the pituitary and immune systems. In C. J. Grossman (ed), *Bilateral Communication Between the Endocrine and Immune System*, Endocrinology and Metabolism: Progress in Research and Clinical Practice, Springer-Verlag, New York, pp 96-144.

Berg, J.M., and Shi, Y. (1996) The galvanization of biology: a growing appreciation for the roles of zinc. Science 271, 1081-1085.

Bettger, W.J., and O'Dell, B.L. (1981) A critical physiological role of zinc in the structure and function of biomembranes. Life Sciences 28, 1425-1438.

Bianchi, F., Rousseaux-Prevost, R., Sautiere, P., and Rousseaux, J. (1992) P2 protamines from human sperm are zinc-finger proteins with one CYS_2/HIS_2 motif. Biochem. Biophys. Res. Comm. 182, 540-547.

Billingsley, G.D., Walter, M.A., Hammond, G.L., and Cox, D.W. (1993) Physical mapping of four serpin genes: alpha-antitrypsin, alpha-antichymotrysin, corticosteroid-binding globulin, and protein C inhibitor, within a 280-kb region on chromosome 14 q 32.1. Am. J. Hum. Genet. 52, 343-353.

Birkenmeier, E.H. and Gordon, J.I. (1986) Developmental regulation of a gene that encodes a cysteine-rich intestinal protein and maps near the murine immunoglobulin heavy chain locus. Proc. Natl. Acad. Sci. USA 83, 2516-2520.

Bissonnette, R.P., Echeverri, F., Mahboubi, A., and Green, D.R. (1992) Apoptotitic cell death induced by c-myc is inhibited by bcl-2. Nature 359, 552-554.

Bogden, J.D. (1995) Studies on micronutrient supplements and immunity in older people. Nutr. Rev. 53, S59-S65.

Bogden, J.D., Bendich, A., Kemp, F.W., *et al.* (1994) Daily micronutrient supplements enhance delayed-hypersensitivity skin test responses in older people. Am. J. Clin. Nutr. 60, 437-447.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., *et al.* (1993) Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74, 597-608.

Bolander, F.F. (1994) Molecular Endocrinology. Academic Press, 2nd edition.

Bonomini, M., Manfrini, V., Cappelli, P., and Albertazzi, A. (1993) Zinc and cell-mediated immunity in chronic uremia. Nephron 65, 1-4.

Bonomini, M., Palmieri, P.F., Evangelista, M., *et al.* (1991) Zinc-mediated lymphocyte energy charge modification in dialysis patients. ASAIO Trans. 37, 387-389.

Borg, L.A., Cagliero, E., Sandler, S., *et al.* (1992) Interleukin-1β increases the activity of superoxide dismutase in rat pancreatic islets. Endocrinology 130, 2851-2857.

Boukaiba, N., Flament, C., Archers, S., *et al.* (1993) A physiological amount of zinc supplementation: effects on nutritional, lipid, and thymic status in an elderly population. Am. J. Clin. Nutr. 57, 566-572.

Bovering, K.E., and Dean, R.T. (1992) Restriction of the participation of copper in radical-generating systems by zinc. Free Radical Res. Commun. 14, 217-225.

Bray. T.M. and Bettger, W. J. (1990) The physiological role of zinc as an antioxidant. Free Rad. Biol. Med. 8, 281-291.

Bremner, I. (1987) Interactions between metallothionein and trace elements. Prog. Food Nutr. Sci. 11, 1-37.

Bremner, I., and Beattie, J.H. (1990) Metallothionein and the trace minerals. Annu. Rev. Nutr. 10, 63-83.

Bremner, I., Morrison, J.M., Wood, A.M., and Arthur, J.R. (1987) Effects of changes in dietary zinc, copper and selenium and of endotoxin administration on metallothionein I concentrations in blood cells and urine in the rat. J. Nutr. 117, 1595-1602.

Bremner, I., and May, P.M. (1989) Systematic interactions of zinc. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 95-108.

Bresnick, E.H., Dalman, F.C., and Pratt, W.B. (1990) Direct stoichiometric evidence that the untransformed Mr 300,000, 9S glucocorticoid receptor is a core unit derived from a larger heteromeric complex. Biochemistry 29, 520-527.

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

Brown, D.G., Sun, X.M., and Cohen, G.M. (1993) Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. J. Biol. Chem. 268, 3037-3039.

Brown, E.D., Chan, W., and Smith, J.C. Jr. (1978) Bone mineralization during a developing zinc deficiency. Proc. Soc. Exp. Biol. Med. 157, 211-214.

Buchingham, J. C., Safieh, B., Singh, S., *et al.* (1992) Interaction between the hypothalamo-pituitary adrenal axis and the thymus in the rat: a role for corticotropin in the control of thymulin release. J. Neuroendocrin. 4, 295-301.

Bunce, G.E. (1989) Zinc in endocrine function. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biolog*, Springer-Verlag, London, pp 249-258.

Bunker, V. W., Hinks, L. J., Stansfield, M. E., *et al.* (1987) Metabolic balance studies for zinc and copper in housebound elderly people and the relationship between zinc balance and leukocyte zinc concentrations. Am. J. Clin. Nutr. 46, 353-359.

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

Burr, R.G. (1973) Blood Zinc in the spinal patient. J. Clin. Path. 26, 773-775.

Butcher, E.C., and Picker, L.J. (1996) Lymphocyte homing and homeostasis. Science 272, 60-66.

Caelles, C., Helmberg, A., and Karin, M. (1994) p53-Dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature 370, 220-223.

Calvano, S.E., Albert, J.D., Legaspi, A., *et al.* (1987) Comparison of numerical and phenotypic leukcyte changes during constant hydrocortisone infusion in normal humans with those in thermally injured patients. Surg. Gynecol. Obstet. 164, 509-520.

Calvano, S.E., de-Riesthal, H.F., Marano, M.A., and Antonacci, A.C. (1988) The decrease in peripheral blood CD4+ T cells following thermal injury in humans can be accounted for by a concomitant decrease in suppressor-inducer CD4+ T cells as assessed using anti-CD45R. Clin. Immunol. Immunopathol. 47, 164-173.

Campbell, K.S., and Cambier, J.C. (1990) B-lymphocyte antigen receptors (mIg) are non-covalently associated with a disulfide-linked, inducibly phosphorylated glycoprotein complex. EMBO J. 9, 441-448.

Carlomagna, M.A., and McMurray, D.N. (1983) Chronic zinc deficiency in rats: its influence on some parameters of humoral and cell-mediated immunity. Nutr. Res. 3, 69-78.

Carson-Jarica, M.A., Schrader, W.T., and O'Malley, B.W. (1990) Steroid receptor family: structure and functions. Endocrine Rev. 11, 201-220.

Cavan, K.R., Gibson, R.S., Grazioso, C.F., *et al.* (1993) Growth and body composition of periurban Guatemalan children in relation to zinc status: a cross-sectional study. Am. J. Clin. Nutr. 57, 334-343.

Chandra, R.R. (1980) *Acrodermatitis enteropathica*: Zinc levels and cell-mediated immunity. Pediatrics 66, 789-791.

Chatterji, D., and Wu, F.Y. (1982) Selective substitution *in vitro* of an intrinsic zinc of *Escherichia coli* RNA polymerase with various divalent metals. Biochemistry 21, 4651-4656. Dreosti, I.E., Record, I.R., Manuel, S.J., and Buckley, R.A. (1981) High plasma zinc levels following oral dosing in rats and the incorporation of 3H-thymidine into deoxyribonucleic acid in rat fetuses. Res. Commun. Chem. Pathol. Pharmacol. 31, 503-513.

Chen, J., Stall, A.M., Herzenberg, L.A., and Herzenberg, L.A. (1990) Differences in glycoprotein complexes associated with IgM and IgD on normal murine B cells potentially enable transduction of different signals. EMBO J. 9, 2117-2124.

Chesters, J.K., and Quarterman, J. (1970) Effects of zinc deficiency on food intake and feeding patterns of rats. Br. J. Nutr. 24, 1061-1069.

Chesters, J.K., and Boyne, R. (1991) Nature of the Zn^{2+} requirement for DNA synthesis by 3T3 cells. Exp. Cell. Res. 192, 631-634.

Chesters, J.K. (1978) Biochemical functions of zinc in animals. Wld. Rev. Nutr. Diet. 32, 135-164.

Chesters, J.K., Boyne, R., Petrie, L., and Lipson, K.E. (1995) Role of the promoter in the sensitivity of human thymidine kinase to lack of Zn^{2+}. J. Biochem. 308, 659-664.

Chesters, J.K. (1989) Biochemistry of zinc in cell division and tissue growth. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 109-128.

Chesters, J.K., Petrie, L., and Lipson, K.E. (1993) Two zinc-dependent steps during G1 to S phase transition. J. Cell. Physiol. 155, 445-451.

Chesters, J.K., Petrie, L. and Travis, J. (1990) A requirement for Zn^{2+} for the induction of thymidine kinase but not ornithine decarboxylase in 3T3 cells stimulated from quiescence. Biochem. J. 272, 525-527.

Chesters, J.K (1972) The role of zinc ions in the transformation of lymphocytes by phytohaemagglutinin. Biochem. J. 130, 133-139.

Chesters, J.K. (1991) Trace element-gene interactions with particular reference to zinc. Proc. Nutr. Soc. 50, 123-129.

Chesters, J.K. (1975) Comparison of the effects of zinc deprivation and actinomycin D on ribonucleic acid synthesis by stimulated lymphocytes. Biochem. J. 150, 211-218.

Chesters, J.K., Petrie, L., and Lipson, K.E. (1993) Two zinc-dependent steps during G_1 to S phase transition. J. Cell. Physiol. 155, 445-451.

Chesters, J.K. (1992) Trace element-gene interactions. Nutr. Rev. 8, 217-223.

Cidlowski, J.A., King, K.L., Evans-Storms, R.B., *et al.* (1996) The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. Recent Prog. Horm. Res. 51, 457-491.

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

Claman, H. (1972) Corticosteroids and lymphoid cells. New Engl. J. Med. 287, 388-398.

Clegg, M.S., Keen, C.L., and Hurley, L.S. (1989) Biochemical pathologies of zinc deficiency. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 129-145.

Coffman, R.L., and Weissman, I.L. (1981) B220: A B cell specific marker at the T200 glycoprotein family. Nature 289, 681-683.

Cohen, J.J., and Duke, R.C. (1992) Apoptosis and programmed cell death in immunity. Annu. Rev. Immunol. 10, 267-293.

Cohen, J.J., and Crnic, L.S. (1982) Glucocorticoids, stress, and the immune response. In D.R. Webb (ed), *Immunopharmacology and the Regulation of Leukocyte Function*, Dekker, New York, pp 61-91.

Cohen, J.J. (1992) Glucocorticoid-induced apoptosis in the thymus. Sem. Immunol. 4, 363-369.

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

Cohen, J.J. (1991) Programmed cell death in the immune system. Adv. Immunol. 50, 55-85.

Coleman, J.E. (1992) Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu. Rev. Biochem. 61, 897-946.

Coligan, J.E., Krusibeek, A.M., Margulies, D.H., *et al.* (1991a) ACK lysing buffer. In: *Current Protocols in Immunology. National Institutes of Health.* Vol. 1, Greene Publishing Associates and Wiley, Interscience, pp 3.1.5.

Coligan, J.E., Krusibeek, A.M., Margulies, D.H., *et al.* (1991b) Trypan blue exclusion test of viability. In *Current Protocols in Immunology*, National Institute of Health, Vol. 1, Greene Publishing Associates and Wiley, Interscience, pp A.3.3.

Compton, M. M., and Cidlowski, J. A. (1992) Thymocyte apoptosis: a model of programmed cell death. Trends. Endocrinol. Metab. 3, 17-23.

Compton, M. M., Caron, L-A. M., and Cidlowski, J. A. (1987) Glucocorticoid action on the immune system. J. Steroid. Biochem. 27, 201-208.

Consolini, R., Cei, B., Cini, P., *et al.* (1986) Circulating thymic hormone activity in young cancer patients. Clin. Exp. Immunol. 66, 173-180.

Cook-Mills, J., and Fraker, P.J. (1993a) Functional capacity of residual lymphocytes from zinc deficient adult mice. Brit. J. Nutr. 69, 835-848.

Cook-Mills, J.M., and Fraker, P.J. (1993b) Optimization of peroxide production by resident macrophages. J. Leukoc. Biol. 53, 205-207.

Cooper, M.D., Mulvaney, D., Coutinho, A., and Cazenave, P.A. (1986) A novel cell surface molecule on early B-lineage cells. Nature 321, 616-618.

Cory, S. (1995) Regulation of lymphocyte survival by the bcl-2 gene family. Annu. Rev. Immunol. 13, 513-543.

Coto, J.A., Hadden, E.M., Sauro, M., *et al.* (1992) Interleukin 1 regulates secretion of zinc-thymulin by human thymic epithelial cells and its action on T-lymphocute proliferation and nuclear protein kinase C. Proc. Natl. Acad. Sci. 89, 7752-7756.

Coudray, C., Boucher, F., Richard, M.J., *et al.* (1991) Zinc deficiency, ethanol, and myocardial ischemia affect lipoperoxidation in rats. Biol. Trace Elem. Res. 30, 103-118.

Cousins, R.J. (1985) Toward a molecular understanding of zinc metabolism. Clin. Physiol. Biochem. 4, 20-30.

Cousins, R.J. (1996) Zinc. *Present Knowledge in Nutrition*, 7th edition, pp 293-306.

Cousins, R.J. (1989) Theoretical and practical aspects of zinc uptake and absorption. In J.A. Laszlo, and F.R. Dintzis (eds), *Mineral Absorption in the Monogastric GI Tract: Chemical, Nutritional and Physiological Aspects*, Plenum, New York, pp 3-12.

Cousins, R.J., and Lee-Ambrose, L.M. (1992) Nuclear zinc uptake and interactions and matallothionein gene expression are influenced by dietary zinc in rats. J. Nutr. 122, 56-64.

Cousins, R.J. (1989) Systemic transport of zinc. In C.F. Mills (ed) *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 79-93.

Cousins, R.J., (1985) Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Phys. Rev. 65, 238-309.

Cousins, R.J. (1983) Metallothionein - aspects related to copper and zinc metabolism. J. Inherit. Metab. Dis. 1, 15-21.

Cousins, R.J. and Leinart, A.S. (1988) Tissue-specific regulation of zinc metabolism and metallothionein genes by interleukin 1. FASEB J. 2, 2884-2890.

Cupps, T.R., and Fauci, A.S. (1982) Corticosteroid-mediated immunoregulation in man. Immunological Rev. 65, 133-155.

da Silva, J. J. R., and Williams, R. J. P. (eds) (1991) The biological chemistry of the elements: the inorganic chemistry of life, Clarendon Press, Oxford.

Daeschner, C.W., Carpentieri, U., Goldman, A.S., and Haggard, M.E. (1985) Zinc deficiency and blood lymphocyte function with sickle cell disease. Scand. J. Haematol. 35, 186-190.

Dahlman-Wright, K., Wright, A., Carlstedt-Duke, J., and Gustafsson, J.A. (1992) DNA-binding by the glucocorticoid receptor: a structural and functional analysis. J. Steroid Biochem. Mol. Biol. 41, 249-272.

Dahlman-Wright, K., Grandien, K., Nilsson, S., *et al.* (1993) Protein-protein interactions between the DNA-binding domains of nuclear receptors influence DNA-binding. J. Steroid Biochem. Mol. Biol. 45, 239-250.

Daniel W.W. (1987) Biostatistics: A foundation for analysis in the health sciences. Fourth edition. John Wiley & Sons, Inc., New York.

Dantzer, R., and Kelley, K.W. (1989) Stress and immunity: an integral view of relationship between the brain and the immune system. Life Sci. 44, 1995-2008.

Dardenne, M., Pleau, J.M., Nabarra, B., *et al.* (1982) Contribution of zinc and other metals to the biological activity of serum thymic factor. Proc. Natl. Acad. Sci. 79, 5370-5373.

De, S.K., McMaster, M.T., and Andrews, G.K. (1990) Endotoxin induction of murine metallothionein gene expression. J. Biol. Chem. 265, 15267-15274.

DelRey, A., Besedovsky, H., and Sorkin, E. (1984) Endogenous blood levels of corticosterone control the immunologic cell mass and B cell activity in mice. J. Immunol. 133, 572-575.

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

DePasquale-Jardieu, P., and Fraker, P.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, 2650-2655.

DePasquale-Jardieu, P., and Fraker, P.J. (1984) Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. J. Nutr. 114, 1762-1769.

Desiderio, S. (1994) The B cell antigen receptor in B-cell developement. Curr. Opin. Immunol. 6, 248-256.

Dexter, T.M., Allen, T.D., and Lajtha, L.G. (1977) Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. J. Cell Physiol. 91, 335-344.

Dexter, T.M., and Testa, N.G. (1980) *In vitro* methods in haemopoiesis and lymphopoiesis. J. Immunol. Methods 38, 177-190.

Dhabhar, F.S., Miller, A.H., McEwen, B.S., and Spencer, R.L. (1995) Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. J. Immunol. 154, 5511-5527.

Dhabhar, F.S., Miller, A.H., McEwen, B.S., and Spencer, R.L. (1995) Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats. J. Neuroimmunol. 56, 77-90.

Dillaha, C. J., Lorincz, A. L., and Auvik, O. R. (1953) *Acrodermatitis enteropathica*: review of the literature and report of a case successfully treated with diodoquin. J. Am. Med. Assoc. 152, 509-512.

DiSilvestro, R.A., and Carlson, G.P. (1994) Effects of mild zinc deficiency, plus or minus acute phase response, on CCl_4 hepatotoxicity. Free Radic. Biol. Med. 16, 57-61.

Distelhorst, C.W. (1989) Recent insight into the structure and function of the glucocorticoid receptor. J. Lab. Clin. Med. 113, 404-412.

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

Dorshkind, K., Schouest, L., Fletcher, W.H. (1985) Morphologic analysis of long-term bone marrow cultures that support B-lymphopoiesis or myelopoiesis. Cell Tissue Res. 239, 375-382.

Dragone, L.L., Barth, R.K., Sitar, K.L., *et al.* (1995) Disregulation of leukosialin (CD43, Ly48, sialophorin) expression in the B-cell lineage of transgenic mice increases splenic B-cell number and survival. Proc. Natl. Acad. Sci. USA. 92, 626-630.

Duncan, J.R., and Dreosti, I.E. (1976) A proposed site of action for zinc in DNA synthesis. J. Comp. Pathol. 86, 81-85.

Dunn, A.J. (1988) Stress-related changes in cerebral catecholamine and indoleamine metabolism: lack of effect of adrenalectomy and corticosterone. J. Neurochem. 51, 406-412.

Durnam, D.M., and Palmiter, R.D. (1987) Analysis of the detoxification of heavy metal ions by mouse metallothionein. Exs. 52, 457-463.

Duvall, E., and Wyllie, A. H. (1986) Death and the cell. Immunol. Today 7, 115-119.

Ebadi, M., and Iversen, P.L. (1994) Mettalotionein in carcinogenesis and cancer chemotherapy. Gen. Pharmac. 25, 1297-1310.

Ehlich, A., Schaal, S., Gu, H., *et al.* (1993) Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. Cell 72, 695-704.

Elmes, M. (1977) Apoptosis in the small intestine of zinc-deficient and fasted rats. J. Pathol. 123, 219-223.

Endre, L., Beck, F., and Prasad, A.S. (1990) The role of zinc in human health. J. Trace Elem. Exp. Med. 3, 337-375.

Enk, A.H., and Katz, S.I. (1994) Heat-stable antigen is an important costimulatory molecule on epidermal Langerhans cells. J. Immunol. 152, 3264-3270.

Etzel, K.R., Swerdel, M.R., Swerdel, J.N., and Cousins, R.J. (1982) Endotoxin-induced changes in copper and zinc metabolism in the Syrian hamster. J. Nutr. 112, 2363-2373.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., *et al.* (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell 69, 119-128.

Evans, R.M. (1988) The steroid and thyroid hormone recptor superfamily. Science 240, 889-895.

Fabris, N., Mocchegiani, E., and Amadio, L., *et al.* (1984) Thymic hormone deficiency in normal aging and Down's syndrome: is there a primary failure of the thymus? Lancet I, 983-986.

Fadok, V.A., Savill, J.S., Haslett, C., *et al.* (1992b) Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. 149, 4029-35.

Fadok, V.A., Voelker, D.R., Campbell, P.A., *et al.* (1992a) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. 148, 2207-2216.

Fadok, V.A., Laszlo, D.J., Noble, P.W., *et al.* (1993) Particle digestibility is required for induction of the phosphatidylserine recognition mechanism used by murine macrophages to phagocytose apoptotic cells. J. Immunol. 151, 4278-4285.

Faict, D., Ceupens, J., and DeMoor, P. (1985) Transcortin modulates the effects of cortisol on mitogen-induced lymphocyte proliferation and immunoglobulin production. J. Steroid Biochem. 23, 553-555.

Faires, L.M. (1982) Inductively Coupled plasma: principles and horizons. Am. Lab. Nov. 18-22.

Falchuck, K.H., Ulpino, L., Mzus, B., and Vallee, B.L. (1977) *E. gracilis* RNA polymerase I: a zinc metalloenzyme. Biochem. Biophys. Res. Commun. 74, 1206-1210.

Falchuk, K.H., Fawcett, D., and Vallee, B.L. (1975) Role of zinc in cell division of *Euglena gracilis*. J. Cell. Sci. 17, 57-68.

Falchuk, K.H., Hardy, C., Ulpino, L., and Vallee, B.L. (1978) RNA metabolism, manganese, and RNA polymerases of zinc sufficient and zinc deficient *Euglena gracilis*. Proc. Natl. Acad. Sci. USA 75, 4175-4179.

Fauci, A.S., and Dale, D.C. (1974) The effect of *in vivo* hydrocortisone on subpopulations of human lymphocytes. J. Clin. Invest. 53, 240-246.

Feldman, D., Funder, J.W., and Edelman, I.S. (1973) Evidence for a new class of carticosterone receptors in the rat kidney. Endocrinology 92, 1429-1441.

Felicia, Y.H., Wu, C.W., and Wu, W. (1987) Zinc in DNA replication and transcription. Ann. Rev. Nutr. 7, 251-272.

Fenwick, P.K., Aggett, P.J., Macdonald, D., *et al.* (1990a) Zinc deficiency and zinc repletion: effect on the response of rats to infection with *Trichinella spiralis*. Am. J. Clin. Nutr. 52, 166-172.

Fenwick, P.K., Aggett, P.J., Macdonald, D., *et al.* (1990b) Zinc deprivation and zinc repletion: effect on the response of rats to infection with *Strongyloides ratti*. Am. J. Clin. Nutr. 52, 173-177.

Ferguson, S.E., Accavitti, M.A., Wang, D.D., *et al.* (1994) Regulation of RAG-2 protein expression in avian thymocytes. Mol. Cell. Biol. 14, 7298-7305.

Fernandes, G., Nair, M., Onoe, K., *et al.* (1979) Impairment of cell-mediated immunity functions by dietary zinc deficiency. Proc. Natl. Acad. Sci. USA. 76, 457-461.

Flaherty, D.K., McGarity, K.L., Sinzenburger, P., and Panyik, M. (1993) The effect of continuous corticosterone administration on lymphocyte subpopulations in the peripheral blood of the Fischer 344 rat as determined by two color flow cytometric analysis. Immunopharmacol. Immunotoxicol. 15, 583-604.

Flynn, A., Pories, W.J., Strain, W.H., and Hill Jr., V.A. (1973) Zinc deficiency with altered adrenacortical function and its relation to delayed healing. Lancet I, 789-791.

Fowlkes, B.J., and Pardoll, D.M. (1989) Molecular and cellular events of T-cell development. Adv. Immunol. 44, 207-264.

Fraker, P.J., and Telford, W. (1996) Regulation of apoptotic events by zinc. In C. Berdanier (ed), *Nutrition and Gene Expression*, CRC Press, Boca Raton, FL, pp 189-208.

Fraker, P.J., Zwickl, C.M., and Luecke, R.W. (1982) Delayed type hypersensitivity in the zinc deficient adult mice: impairment and restoration of responsitivity to dinitrofluorobenzene. J. Nutr. 112, 309-313.

Fraker, P.J., Gershwin, M.E., Good, R.A., and Prasad, A.S. (1986) Interrelationships between zinc and immune function. Feder. Proc. 45, 1474-1479.

Fraker, P.J., King, L.E., Garvy, B.A., and Medina, C.A. (1993) The immunopathology of zinc deficiency in humans and rodents. In D.M. Klurfeld (ed), *Human Nutrition - A Comprehensive Treatise, Nutrition and Immunology*, Vol. 8, Plenum Press, New York, pp 267-284.

Fraker, P.J., DePasquale-Jardieu, P., Zwickl, C.M., and Leucke, R.W. (1978) Regeneration of T-cell helper function in zinc-deficient adult mice. Proc. Natl. Acad. Sci. USA 75, 5660-5664.

Fraker, P.J., Haas, S.M., and Leucke, R.W. (1977) Effect of zinc deficiency on the immune response of the young adult A/J mouse. J. Nutr. 107, 1889-1895.

Fraker, P.J., Osati-Ashtiani, F., Wagner, M.A., and King, L.E. (1995) Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: a review. J. Am. Coll. Nutr. 14, 11-17.

Fraker, P.J., Caruso, R., and Kierszenbaum, F. (1982) Alteration of the immune and nutritional status of mice by synergy between zinc deficiency and infection with *Trypanosoma cruzi*. J. Nutr. 112, 1224-1229.

Fraker, P.J., Hildebrandt, K., and Luecke, R.W. (1984) Alteration of antibody-mediated responses of suckling mice to T-cell-dependent and independent antigens by maternal marginal zinc deficiency: restoration of responsivity by nutritional repletion. J. Nutr. 114, 170-179.

Freedman, L.P. (1992) Anatomy of the steroid receptor zinc finger region. Endocrine Rev. 13, 129-145.

Freedman, L.P., Luisi, B.F., Korszvn, Z.R., *et al.* (1988) The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature 334, 543-546.

Fukuda, M. (1991) Leukosialin, a major O-glycan-containing sialoglycoprotein defining leukocyte differentation and malignancy. Glycobiology 1, 347-356.

Garvy, B., King, L., Telford, W., *et al.* (1993a) Chronic levels of corticosterone reduces the number of cycling cells of the B-lineage in murine bone marrow and induces apoptosis. Immunology 80, 587-592.

Garvy, B.A., Telford, W.G., King, L.E., and Fraker, P.J. (1993b) Glucocorticoids and irradiation-induced apoptosis in normal murine bone marrow B-lineage lymphocytes as determined by flow cytometry. Immunology 79, 270-277.

Garvy, B.A., and Fraker, P.J. (1991) Suppression of the antigenic response of murine bone marrow B cells by physiological concentrations of glucocorticoids. Immunology 74, 519-523.

Gasull, T., Giralt, M., Hernandez, J., *et al.* (1994) Regulation of metallothionein concentrations in rat brain: effect of glucorticoids, zinc, copper, and endotoxin. Am. J. Physiol. Soc. 266, E760-E767.

Giglio, M.P., Hunter, T., Bannister, J.V., *et al.* (1994) The manganese superoxide dismutase gene of *Caenorhabditis elegans*. Biochem. Mol. Biol. Int. 33, 37-40.

Giroux, E.L., and Henkin, R.I. (1972) Competition for zinc among serum albumin and amino acids. Biochem. Biophys. Acta. 273, 64-72.

Giugliano, R., and Millward, D.J. (1984) Growth and zinc homeostasis in the severely Zn-deficient rat. Br. J. Nutr. 52, 545-560.

Godfrey, D.I., Kennedy, J., Mombaerts, P., *et al.* (1994) Onset of TCRβ rearrangement and role of TCRβ expression during $CD3^-CD4^-CD8^-$ thymocyte differentiation. J. Immunol. 152, 4783-4792.

Godowski, P.J., and Picard, D. (1989) Steroid receptors: How to be both a receptor and a transcription factor. Biochem. Pharmacol. 38, 3135-3143.

Godowski, P.J., Picard, D., and Yamamoto, K.R. (1988) Signal transduction and transcriptional regulation by glucocortoid receptor. Lex A fusion proteins. Science 241, 812-816.

Golden, B.E. (1989) Zinc in cell division and tissue growth: physiological aspects. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Spring-Verlag, London, pp 119-128.

Golden, M.H.N., Jackson, A.A., and Golden, B.E. (1977) Effect of zinc on thymus of recently malnurished children. Lancet II, 1057-1059.

Good, R.A., and Lorenz, E. (1992) Nutrition and cellular immunity. Int. J.Immunopharmacol. 14, 361-366.

Gorodetsky, R., Fuks, Z., Sulkes, A., *et al.* (1985) Correlation of erythrocyte and plasma levels of zinc, copper and iron with evidence of metastatic spread in cancer patients. Cancer 55, 779-787.

Greaves, M.F., Brown, J., Molgaard, H.V., *et al.* (1992) Molecular features of CD34: a hemopoietic progenitor cell-associated molecule. Leukemia 6, 31-36.

Grider, A., and Young, E.M. (1996) The *Acrodermatitis enteropahthica* mutation transiently affects zinc metabolism in human fibroblasts. J. Nutr. 126, 219-224.

Gruber, J., Sgonc, R., Hu, Y. H., Beng, H., *et al.* (1994) Thymocyte apoptosis induced by elevated endogenous corticosterone levels. Eur. J. Immunol. 24, 1115-1121.

Guidos, C.J., Williams, C.J., Wu, G.E., *et al.* (1995) Development of $CD4^+CD8^+$ thymocytes in RAG-deficient mice through a T cell receptor β chain-independent pathway. J. Exp. Med. 181, 1187-1195.

Gulley, M.L., Ogata, L.C., Thorson, J.A., *et al.* (1988) Identification of a murine pan-T cell antigen which is also expressed during the terminal phases of B cell differentiation. J. Immunol. 140, 3751-3757.

Gupta, R.K., Bhattacharya, S.K., Sundar, S., *et al.* (1992) A correlative study of serum zinc and *in vitro* cell mediated immune status in rheumatic heart disease. Acta Cardiologica. 157, 297-304.

Gustafsson, J.A., Carlstedt-Duke, J., Stromstedt, P.E., *et al.* (1990) Structure, function and regulation of the glucocorticoid receptor. In G.H. Sato, and J.L. Stevens (eds), *Molecular Endocrinology and Steroid Action*, Alan R. Liss, Inc., New York, pp 65-80.

Gustafsson, J.A., Carlstedt-Duke, J., Poellinger, L., *et al.* (1987) Biochemistry, molecular biology, and physiology of the glucocorticoid receptor. Endocr. Rev. 8, 185-234.

Guttinger, M., Gassman, M., Amrein, K.E., and Burn, P. (1992) CD45 phosphotyrosine phosphatase and p56lck protein tyrosine kinase: a functional complex crucial in T cell signal transduction. Int. Immunol. 4, 1325-1330.

Hadden, J.W. (1995) The treatment of zinc deficiency is an immunotherapy. Int. J. Immunopharmac. 17, 697-701.

Hadley, M.C. (1992) Chapter 15: Adrenal steriod Hormones. Endocrinology, 3rd edition, Prentice-Hall, Inc., New Jersey, pp 391-429.

Hager, L.J., and Palmiter, R.D. (1981) Transcriptional regulation of mouse liver metallothionein-I gene by glucocorticoids. Nature 291, 340-342.

Hager, L.J., and Palmiter, R.D. (1981) Transcriptional regulation of mouse liver metallothionein-I gene by glucocorticoids. Nature 291, 340-342.

Hahne, M., Wenger, R.H., Vestweber, D., and Nielsen, P.J. (1994) The heat-stable antigen can alter very later antigen 4-mediated adhesion. J. Exp. Med. 179, 1391-1395.

Hambidge, K.M. (1989) Mild zinc deficiency in human subjects. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag , London, pp 281-296.

Hambidge, K.M., Casey, C.E., and Krebs, N.F. (1986) Zinc. In W. Mertz (ed), *Trace Elements in Human and Animal Nutrition*, Vol. 2, 5th ed, Academic Press, New York, pp 1-137.

Hamer, D.H. (1986) Metallothionein. Ann. Rev. Biochem. 55, 913-951.

Hammond, G.L. (1990) Molecular properties of coritcosteroid binding globulin and the sex-steroid binding proteins. Endo. Rev. 11, 65-79.

Hammond, G.L., Smith, C.L., Goping, L.S., *et al.* (1987) Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. Proc. Natl. Acad. Sci. USA 84, 5153-5157.

Hammond, G.L., Smith, C.L., and Underhill, D.A. (1991) Molecular Studies of corticosteroid binding globulin structure, biosynthesis and function. J. Steroid Biochem. Mol. Biol. 40, 755-762.

Han, O., Failla, M.L., Hill, A.D., *et al.* (1994) Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. J. Nutr. 124, 580-587.

Hard, T., Dahlman, K., Carstedt-Duke, J., *et al.* (1990) Cooperativity and specificity in the interactions between DNA and the glucocorticoid receptor DNA-binding domain. Biochemistry 29, 5358-5364.

Hardy, R.R., Carmack, C.E., Shinton, S.A., *et al.* (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med. 173, 1213-1225.

Hayashi, J., Medlock, E.S., and 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.

Hedrick, S.M., and Eidelman, F.J. (1993) T lymphocyte antigen receptors. Fund. Immun. 3rd edition, 383-413.

Heimfeld, S., and Weissman, I.L. (1992) Characterization of several classes of mouse hematopoietic progenitor cells. Curr. Top. Microbiol. Immunol. 177, 95-105.

Held, D.D. and Hoekstra, W.G. (1984) The effects of zinc deficiency on turnover of cadmium-metallothionein in rat liver. J. Nutr. 114, 2274-2282.

Hempe, J.M. and Cousins, R.J. (1991) Cysteine-rich intestinal protein binds zinc during transmucosal zinc transport. Proc. Natl. Acad. Sci. USA 88, 9671-9674.

Hempe, J.M., and Cousins, R.J. (1992) Cysteine-rich intestinal protein and intestinal metallothionein: an inverse relationship as a conceptual model for zinc absorption in rats. J. N. 122, 89-95.

Henkin, R.I. (1974) On the role of adrenocorticosteroids in the control of zinc and copper metabolism. In W.G. Hoekstra, J.W. Suttie, H.E. Ganther, and W. Mertz (eds), *Trace Element Metabolism in Animals-II. Proceedings of the second International symposium on Trace Element Metabolism in Animals held in Madison, Wisconsin*, University Park Press, Baltimore, pp 647-651.

Hermann, G., Tovar, C.A., Beck, F.M., and Sheridan, J.F. (1994) Kinetics of glucocorticoid response to restraint stress and/or experimental influenza viral infection in two inbred strains of mice. J. Neuroimmunol. 49, 25-33.

Hermeking, H., and Eick, D. (1994) Mediation of c-myc -induced apoptosis by p53. Science, 265, 2091-2093.

Hicks, S.E., and Wallwork, J.C. (1987) Effect of dietary zinc deficiency on protein synthesis in cell-free systems isolated from rat liver. J. Nutr. 117, 1234-1240.

Hill, A.D., Naama, H.A., Gallagher, H.J., *et al.* (1995) Glucocorticoids mediate macrophage dysfunction in protein calorie malnurtion. Sugery 118, 130-137.

Hoadley, J.E., Leinart, A.S., and Cousins, R.J. (1988) Relationship of ^{65}Zn absorption kinetics to intestinal metallothionein in rats: effects of zinc depletion and fasting. J. Nutr. 118, 497-502.

Hoadley, J.E., and Cousins, R.J. (1987) Cellular mechanisms for intestinal zinc absorption. Fed. Proc. 46, 598-604.

Hombach, J., Leclercq, L., Radbruch, A., *et al.* (1988) A novel 34 kD protein co-isolated with IgM molecule in surface IgM expressing cells. EMBO J. 7, 3451-3456.

Hombach, J., Lottspeich, F., and Reth, M. (1990) Identification of the genes encoding the Igα and Igβ components of the IgM antigen receptor complex by amino-terminal sequencing. Eur. J. Immunol. 20, 2795-2799.

Homo-Delarche, F., and Dardenne, M. (1993) The neuroendocrine-immune axis. Springer Semin. Immunopathol. 14, 221-238.

Hoovers, J.M.N., Mannens, M.J.R., Bliek, J., *et al.* (1992) High-resolution localization of 69 potential human zinc finger protein genes: a number are clustered. Genomics 12, 254-263.

Hough, M.R., Takei, F., Humphries, R.K., and Kay, R. (1994) Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. J. Exp. Med. 179, 177-184.

Hough, M.R., Chappel, M.S., Sauvageau, G., *et al.* (1996) Reduction of early B lymphocyte precursors in transgenic mice overexpressing the murine heat-stable antigen. J. Immunol. 156, 479-488.

Housely, P.R., Sanchez, E.R., Danielson, M., *et al.* (1990) Evidence that the conserved region in the steroid binding domain of the glucocorticoid receptor is required for both optimal binding of hsp90 and protection from proteolytic cleavage. J. Biol. Chem. 265, 12778-12781.

Hsu, B.R.S., Siiteri, P.K., and Kuhn, R.W. (1986) Ineractions between corticosteroid-binding globulin (CBG) and target tissues. Binding proteins of steroid hormones. Colloque INSERM/ John Libbey Evortext Ltd. 149, 577-591.

Hubbe, M., and Altevogt, P. (1994) Heat-stable antigen-CD24 on mouse T lymphocytes: evidence for a costimulatory function. Eur. J. Immunol. 24, 731-737.

Huber, K.L., and Cousins, R.J. (1993) Metallothionein expression in rat bone marrow is dependent on dietary zinc but not dependent on interleukin-1 or interleukin-6. J. Nutr. 123, 642-648.

Huckle, J.W., Morby, A.P., Turner, J.S., and Robinson, N.J. (1993) Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions. Molec. Microbiol. 7, 177-187.

Hutchison, K.A., Matic, G., Czar, M.J., *et al.* (1992) DNA-binding and non-DNA-binding forms of the transformed glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. 41, 715-718.

Hutchison, K.A., Czar, M.J., and Pratt, W.B. (1992) Evidence that the hormone-binding domain of the mouse glucocorticoid receptor directly represses DNA binding activity in a major portion of receptors that are "misfold" after removal of hspqo. J. Biol. Chem. 267, 3190-3195.

Ikuta, K., Uchida, N., Friedman, J., and Weissman, I.L. (1992) Lymphocyte development from stem cells. Annu. Rev. Immunol. 10, 759-783.

Iwata, M., Hanaloka, S., and Sato, K. (1991) Rescue of thymocytes and T-cell hybridomas from glucocorticoid induced apoptosis by stimulation via the T cell receptor/CD3 complex: Possible *in vitro* model for positive selection of the T cell repertoire. Eur. J. Immunol. 21, 643-648.

Jackson, M.J. (1989) Physiology of Zinc: General aspects. In C.F. Mills (ed), *Human Nutrition Reviews, Zinc in Human Biology*, Springer-Verlag, London, pp 1-14.

Jacobsen, K., Prasad, V.S., Sidman, C.L., and Osmond, D.G. (1994) Apoptosis and macrophage-mediated deletion of precursor B cells in the bone marrow of Eμ-myc transgenic. Blood 84, 2784-2794.

Jardieu, P., and Fraker, P.J. (1990) Influence of zinc deficiency on the magnitude kinetics, and affinity of the response to trinitrophenylated (TNP) lipopolysaccharide in TNP-ficol in adult mice. J. Trace Elements Exp. Med. 3, 1-11.

Jaya Rao, K.S., Srikantia, S.G., and Gopalan, C. (1968) Plasma cortical levels in protein-calorie malnutrition. Arch. Dis. Child. 43, 365-367.

Jenkinson, E.J., Anderson, G., and Owen, J.J.T. (1992) Studies on T cell maturation on defined thymic stromal cell populations *in vitro*. J. Exp. Med. 176, 845-853.

Jerrells, T.R., Marietta, C.A., Weight, F.F., and Eckardt, M.J. (1990) Effects of adrenalectomy on ethanol-associated immunosuppression. Int. J. Immunopharmacol. 12, 435-442.

Kagi, J.H., and Schaffer, A. (1988) Biochemistry of metallothionein. Biochemistry 27, 8509-8515.

Karasuyama, H., Rolink, A., Shinkai, Y., *et al.* (1994) The expression of $V_{pre-B}/\lambda 5$ surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. Cell 77, 133-143.

Karin, M., and Herschmann, H.R. (1981) Induction of metallothionein in Hela cells by dexamethasone and zinc. Eur. J. Biochem. 113, 267-272.

Kashiwamura, S.I., Koyama, T., Matsuo, T., *et al.* (1990) Structure of the murine mb-1 gene encoding a putative surface IgM-associated molecule. J. Immunol. 145, 337-343.

Kawauchi, K., Lazarus, A.H., Rapoport, M.J., *et al.* (1994) Tyrosine kinase and CD45 tyrosine phosphatase activity mediate p21ras activation in B cells stimulated through the antigen receptor. J. Immunol. 152, 3306-3316.

Kay, J., Cryer, A., Darke, B.M., *et al.* (1991) Naturally occurring and recombinant metallothioneins: structure, immunoreactivity and metal-binding functions. Int. J. Biochem. 23, 1-5.

Keen, C.L., and Gershwin, M.E. (1990) Zinc deficiency and immune function. Annu. Rev. Nutr. 10, 415-431.

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

Kenny, A.J., Stephenson, S.L., and Turner, A.J. (1987) In A.J. Kenny, and A.J. Turner (eds), *Mammalian Ectoenzymes*, Elsevier, Amsterdam, pp 169-210.

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

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer. 26, 239-257.

Kimball, S.R., Chen, S.J., Risica, R., *et al.* (1995) Effects of zinc deficiency on protein synthesis and expression of specific mRNAs in rat liver. Metabolism 44, 126-133.

Kincade, P.W. (1981) Formation of B lymphocytes in fetal and adult life. Adv. Immunol. 31, 177-245.

King, L.E., Osati-Ashtiani, F., and Fraker, P.J. (1995) Depletion of cells of the B lineage in the bone marrow of zinc-deficient mice. Immunology 85, 69-73.

King, L.E., and Fraker, P.J. (1991) Flow cytometric analysis of the phenotypic distribution of splenic lymphocytes in zinc-deficient adult mice. J. Nutr. 121, 1433-1438.

Kisielow, P., Bluthmann, H., Staerz, V.D., *et al.* (1988) Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+CD8^+$ thymocytes. Nature 333, 742-746.

Kisielow, P. (1995) Apoptosis in intrathymic T-cell developement. In C.D. Gregory (ed), *Apoptosis and the Immune Response*, Willey-Liss, Inc., New York, pp 13-53.

Kitamura, D., Roes, J., Kuhn, R., and Rajewsky, K. (1991) A B-cell deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature 350, 423-426.

Kitson, R.P., Brunson, K.W., Miller, C.A., and Goldfarb, R.H. (1994) Neuroendocrine modulation of tumor metastasis. *In-Vivo*. 8, 803-806.

Klug, A., and Schwabe, J.W.R. (1995) Zinc fingers. FASEB J. 9, 597-604.

Klug, A., and Rhodes, D. (1987) Zinc fingers: A novel protein motif for nucleic acid recognition. Trends Biochem. Sci. 12, 464-469.

Koch, B., Lutz, B., Briand, B., and Mialhe, C. (1976) Heterogeneity of pituitary glucocorticoid binding evidence for a transcortia-like compound. Biochem. Biophys. Acta. 44, 497-507.

Koretzky, G.A., Kohmetscher, M.A., Kkadleck, T., and Weiss, A. (1992) Restoration of T cell receptor-mediated signal transduction by transfection of CD45 cDNA into a CD45-deficient variant of the Jurkat T cell line. J. Immunol. 149, 1138-1142.

Korsmeyer, S.J. (1995) Regulators of cell death. Trends Genet. 11, 101-105.

Kraft, D.L., Weissman, I.L., and Waller, E.K. (1993) Differentiation of $CD3^-4^-8^-$ human fetal thymocytes *in vivo*: characterization of a $CD3^-4^+8^-$ intermediate. J. Exp. Med. 178, 265-277.

Kraujelis, K., and Desrius, A. (1989) Transcortin biosynthesis and intracellular distribution of rat liver polyribosomes synthesizing transcortin. J. Steriod. Biochem. 32, 121-125.

Kruisbeek, A.M. (1993) Development of $\alpha\beta$ T-cells. Curr. Opin. Immunol. 5, 227-234.

Kuby, J. (1992) Organization and expression of immunoglobulin genes. In *Immunology*, W.H. Freeman and Company, New York, pp 165.

Kuvibidila, S., Yu, L., Ode, D., and Warrier, R.P. (1993) The immune response in protein-energy malnutrition and single nutrient deficiencies. In D.M. Klurfeld (ed), *Human Nutrition. A Comprehensive Treatise*, Vol. 8., *Nutrition and Immunology*, Plenum Press, New York, pp 121-155.

LaFond, R.E., Kennedy, S.W., Harrison, R.W., and Villee, C.A. (1988) Immunocyte chemical localization of glucocorticoid receptors in cells, cytoplasts and nucleoplasts. Exp. Cell Res. 175, 52-62.

Laherty, C.D., Hu, H.M., Opipari, A.W., *et al.* (1992) The epstein-barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor κB. J. Bio. Chem. 267, 24157-24160.

Lan, N.C., Karin, M., Nguyen, T., *et al.* (1984) Mechanisms of glucocorticoid hormone action. J. Steroid Biochem. 20, 77-88.

Landau, N.R., Schatz, D.G., Rosa, M., and Baltimore, D. (1987) Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol. Cell. Biol. 7, 3237-3243.

Lapointe, M.C., and Baxter, J.D. (1989) Molecular biology of glucocarticoid hormone action. In R.P. Scheimer, H.N. Claman, and A. Oronsky (eds), *Anti-inflammatory Steroid Action - Basic and Clinical Aspect,* Academic Press, Inc., pp 3-29.

Lebeau, M.C., Binart, N., Cadepond, F., *et al.* (1994) Steroid receptor associated proteins: heat shock protein 90 and p59 immunophilin. In V.K. Moudgil (ed), *Steroid Hormone Receptors: Basic and Clinical Aspects*, Birkhäuser, Boston, pp 261-280.

Li, Y-S., Hayakawa, K., and Hardy, R.R. (1993) The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. 178, 951-960.

Licastro, F., Mocchegiani, E., Zannotti, M., *et al.* (1992) Zinc affects the metabolism of thyroid hormones in children with Downs syndrome: normalization of thyroid stimulating hormone and of reversal triiodotheronine plasmic levels by deitary zinc supplementation. Intern. J. Neuroscience 56, 259-268.

Lifschitz, M.D., and Henkin, R.I. (1971) Circadian variation in copper and zinc in man. J. Appl. Physiol. 31, 88-92.

Liu, Y., Jones, B., Brady, W., *et al.* (1992) Co-stimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. Eur. J. Immunol. 22, 2855-2859.

Liu, Y.J., Joshua, D.E., Williams, G.T., *et al.* (1989) Mechanism of antigen-driven selection in germinal centers. Nature 342, 929-931.

Lönnerdal, B. (1989) Intestinal absorption of zinc. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag , London, pp 33-35.

Lotem, J., and Sachs, L. (1995) Reguulation of bcl-2, bcl-X_L and bax in the control of apoptosis by hematopoietic cytokines and dexamethasone. Cell Growth Differ. 6, 645-653.

Lowy, M.T. (1989) Quantification of type I and II adrenal steroid receptors in neuronal, lymphoid and pituitary tissues. Brain Res. 503, 191-197.

Luisi, B.F., Xu, W.X., Otwinowski, Z., *et al.* (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352, 497-505.

Ma, A., Pena, J.C., Chang, B. *et al.* (1995) Bcl-x regulates the survival of double-positive thymocytes. Proc. Natl. Acad. Sci. USA 92, 4763-4767.

Macapinlac, M.P. (1966) Some characteristics of zinc deficiency in the albino rat. In A.S. Prasad (ed), *Zinc Metabolism*, Thomas, Illinois, pp 142-165.

Madan, A.P. and DeFranco, D.B. (1993) Bidirectional transport of glucocorticoid receptors across the nuclear envelope. Proc. Natl. Acad. Sci. USA 90, 3588-3592.

Mahajan, S. K. (1988) Zinc metabolism in uremia. Int. J. Artif. Organs, 11, 223-228.

Maldonado, M.D., Venturali, A., Franco, A., and Nunez-Roldan, A. (1991) Specific changes in peripheral blood lymphocyte phenotype from probable origin of the thermal injury-related lymphocytopenia. Burns 17, 188-192.

Marcos, M.A.R., Godin, I., Cumano, A., *et al.* (1994) Developmental events from hemopoietic stem cells to B cell populations and Ig repertoires. Immunol. Rev. 137, 155-171.

Martin, S.J., Mazdai, G., Strain, J.J., *et al.* (1991) Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. Clin. Exp. Immunol. 83, 338-343.

Marx, J. (1995) How the glucocorticoids suppress immunity. Science 270, 232-233.

Masters, D.G., Keen, C.L., Lonnerdal, B., and Hurley, L.S. (1983) Zinc deficiency teratogenicity: the protective role of maternal tissue catabolism. J. Nutr. 113, 905-912.

Matsuo, T., Nomura, J., Kuwahara, K., *et al.* (1993) Cross-linking of B cell receptor-related MB-1 molecule induces protein tyrosine phosphorylation in early B lineage cells. J. Immunol. 150, 3766-3775.

Matsuuchi, L., Gold, M.R., Travis, A., *et al.* (1992) The membrane IgM-associated proteins MB-1 and Ig-B are sufficient to promote surface expression of a particularly functional B-cell antigen receptor in a nonlymphoid cell line. Proc. Natl. Acad. Sci. USA 89, 3404-3408.

McConkey, D.J., Nicotera, P., Hartzell, P., *et al.* (1989) Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. Arch. Biochem. Biophys. 269, 365-370.

McConkey, D.J., Aguilar-Santelises, M., Hartzell, P., *et al.* (1991) Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. J. Immunol. 146, 1072-1076.

Meisfield, R.L. (1990) Molecular genetics of corticosteroid action. Annu. Rev. Respir. Dis. 141, 11-17.

Melchers, F., Rolink, A., Grawunder, U., *et al.* (1995) Positive and negative selection events during B lymphopoiesis. Curr. Opin. Immunol. 7, 214-227.

Melchers, F., Karasuyama, H., Haasner, D., *et al.* (1993) The surrogate light chain in B-cell developement. Immunol. Today 14, 60-68 .

Menard, P., McCormick, C.C., and Cousins, R.J. (1981) Regulation of intestinal metallothionein biosynthesis in rats by dietary zinc. J. Nutr. 111, 1358-1361.

Merino, R., Ding, L., Veis, D.J., *et al.* (1994) Developmental regulation of the Bcl-2 protein and susceptibility to cell death in B lymphocytes. EMBO J. 13, 683-691.

Miller, A.H., Spencer, R.L., and Trestman, R.L. (1991) Adrenal steroid receptor activation *in vivo* and immune function. Am. J. Physiol. 261, E126-E131.

Miller, A.H., Spencer, R.L., Stein, M., and McEwen, B.S. (1990) Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone. Am. J. Physiol. 259, E405-E412.

Miller, W.L. (1988) Molecular biology of steroid hormone synthesis. Endocr. Rev. 9, 295-318.

Miller, J., McLachlan, A.D., and Klug, A. (1985) Repetitive zinc-binding domains in the protein transcription factor III A from *Xenopus oocytes*. EMBO J. 4, 1609-1614.

Miller, A.H., Spencer, R.L., Hassett, J., *et al.* (1994) Effects of selective type I and II adrenal steroid against an immune cell distribution. Endocrinology 135, 1934-1944.

Mills, C.F. (1989) The biological significance of zinc for man: problems and prospects. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 371-381.

Minkus, T.M., Koski, K.G., and Scott, M.E. (1992) Marginal zinc deficiency has no effect on primary or challenge infections in mice with *Heligmosomoides polygyrus* (Nematoda). J. Nutr. 122, 570-579.

Misener, V., Downey, G.P., and Jongstra, J. (1991) The immunoglobulin light chain related protein λ_5 is expressed on the surface of mouse pre-B cell lines and can function as a signal transducing molecules. Int. Immunol. 3, 1129-1136.

Mitchell, J.B., and Meaney, M.J. (1991) Effects of corticosterone on response consolidation and retrieval in the forced swim test. Behav. Neurosci. 105, 798-803.

Mocchegiani, E., Paolucci, P., Granchi, D. *et al.* (1994) Plasma zinc level thymic hormone activity in young cancer patients. Blood 83, 749-757.

Mocchegiani, E., Provinciali, M., DiStefano, G., *et al.* (1995) Role of the low zinc bioavailability on cellular immune effectiveness in cystic fibrosis. Clin. Immunol., Immunopathol. 75, 214-224.

Moore, T., Huang, S., Terstappen, L.W.M.M., *et al.* (1994) Expression of CD43 on murine and human pluripotent hematopoietic stem cells. J. Immunol. 153, 4978-4987.

Moore, M.E.C., Moran, J.R., and Greene, H.L. (1984) Zinc supplementation in lactating women-evidence for mammary control of zinc secretion. J. Pediatr. 105, 660-672.

Morris, R.G., Hargreaves, A.D., Duvall, E., and Wyllie, A.J. (1984) Hormone-induced cell death: surface changes in thymocytes undergoing apoptosis. Am. J. Pathol. 115, 426-436.

Moulder, K., and Steward, M.W. (1989) Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody. Clin. Exp. Immunol. 77, 269-274.

Moynahan, E.J. (1974) *Acrodermatitis enteropathica*: a lethal inherited human zinc deficiency disorder. Lancet II, 399-400.

Moynahan, E.J., and Barnes, P.M. (1973) Zinc deficiency and a synthetic diet for lactose tolerance. Lancet I, 676-677.

Munck, A., Guyre, P.M., and Holbrook, N.J. (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr. Rev. 5, 25-44.

Munck, A., and Crabtree, G.R. (1981) Glucocorticoid-induced lymphocyte death. In I. D. Bowen and R. A. Lockshin (eds), *Cell Death in Biology and Pathology*, Chapman and Hall, London, pp 329-359.

Murphy, K.M., Heimberger, A.B., and Loh, D.Y. (1990) Induction by antigen of intrathymic apoptoxsis of CD4+ CD8+ TCR thymocytes *in vivo*. Science 250, 1720-1723.

Muzzioli, M., Mocchegiani, E., Bressani, N., *et al.* (1992) *In vitro* restoration by thymulin of NK activity of cells from old mice. Int. J. Immunopharmacol. 14, 57-61.

Nagel, W.W., and Vallee, B.L. (1995) Cell cycle regulation of metallothionein in human colonic cancer cells. Proc. Natl. Acad. Sci. USA 92, 579-583.

Nagel, W.W., and Vallee, B.L. (1995) Cell cycle regulation of metallothionein in human colonic cancer cells. Proc. Natl. Acad. Sci. USA 92, 579-583.

Nakayama, K., Nakayama, K., Negishi, I., *et al.* (1993) Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science 261, 1584-1588.

Naveh, Y., Lee-Ambrose, L.M., Samuelson, D.A., and Cousins, R.J. (1993) Malabsorption of zinc in rats with acetic acid-induced enteritis and colitis. J. Nutr. 123, 1389-1395.

Nawar, O., Akridge, R.E., Hassan, E., *et al.* (1992) The effect of zinc deficiency on granuloma formation, liver fibrosis, and antibody responses in experimental *schistosomiasis*. Am. J. Trop. Med. Hyg. 47, 383-389.

Neggers, Y.H., Cutter, G.R., Acton, R.T., *et al.* (1990) A posistive association between maternal serum zinc concentration and birth weight. Am. J. Clin. Nutr. 51, 678-684.

Nicotera, P., and Ross, A.D. (1994) Nuclear Ca^{2+}: physiological regulation and role in apoptosis. Mol. Cell. Biochem. 135, 89-98.

Nieto, M.A., Gonzalez, F., Gamban, F., *et al.* (1992) Apoptosis in human thymocytes after treatement with glucocorticoids. Clin. Exp. Immunol. 88, 341-344.

Nixon D.E., Moyer T.P., Johnson P., *et al.* (1986) Routine measurement of calcium, magnesium, copper, zinc and iron in urine and serum by inductively coupled plasma emission spectroscopy. Clin. Chem. 32, 1660-1665.

Nomura, J., Matsuo, T., Kubota, E., *et al.* (1991) Signal transmission through the B cell specific MB-1 molecule at the pre-B cell stage. In. Immunol. 3, 117-126.

Nunez, G., Merino, R., Grillot, D., and Gonzalez-Garcia, M. (1994) Bcl-2 and Bcl-x: regulatory switches for lymphoid death and survival. Immunol. Today 15, 582-588.

Nyberg, L., Marekeov, L.N., Jones, I., *et al.* (1990) Characterization of the murine corticosteroid binding globulin: variations between mammalian forms. J. Steroid Biochem. 35, 61-65.

Odeh, M. (1992) The role of zinc in acquired immunodeficiency syndrome. J. Inter. Med. 231, 463-469.

Okamoto, M., Morishita, M., Setoguchi, C., and Nakata, K. (1993) Restorative effect of short term administration of thymulin on thymus-dependent antibody production in restraint-stressed mice. Int. J. Immunopharmacol. 15, 757-762.

Orava, M., Zhao, X.F., Leiter, E., and Hammond, G.L. (1994) Structure and chromosomal location of the gene encoding mouse corticosteroid-binding globulin: strain differences in coding sequence and steroid-binding activity. Gene 144, 259-264.

Organ, B.C., Antonacci, A.C., Chiao, J., *et al.* (1989) Changes in lymphocyte number and phenotype in seven lymphoid compartments after thermal injury. Ann. Surg. 210, 78-89.

Osmond, D.G. (1986) Population dynamics of bone marrow B-lymphocytes. Immunol. Rev. 93, 103-124.

Osmond, D.G. (1990) B cell developement in the bone marrow. Semin. Immunol. 2, 173-180.

Osmond, D.G. (1991) Proliferation kinetics and lifespan of B cells in central and peripheral lymphiod organs. Curr. Opin. Immunol. 3, 179-185.

Osmond, D.G. (1993) Production and selection of B-lymphocytes in bone marrow: lymphostromal interactions and apoptosis in normal, mutant and transgenic mice. Adv. Exp. Med. Biol. 355, 15-20.

Osmond, D.G., Rico-Vargas, S., Valenzona, H., *et al.* (1994) Apoptosis and macrophage-mediated cell deletion in the regulation of B-lymphapoiesis in mouse bone marrow. Immunol. Rev. 142, 209-230.

Oteiza, P.I., Olin, K.L., Fraga, C.G., and Keen, C.L. (1995) Zinc deficiency causes oxidative damage to proteins, lipids and DNA in rat testes. J. Nutr. 125, 823-829.

O'Dell, B.L., and Reeves, P.G. (1989) Zinc status and food intake. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 173-181.

Pai, L.H., and Prasad, A.S. (1988) Cellular zinc in patients with diabetes mellitus. Nutr. Res. 8, 889-897.

Pallant, A., Eskenazi, A., Mattei, M.G., *et al.* (1989) Characterization of cDNAs encoding human leukosialin and localization of the leukosialin gene to chromosome 16. Proc. Natl. Acad. Sci. USA 86, 1328-1332.

Palmiter, R.D., and Findley, S.D. (1995) Cloning and Functional characterization of a mammalian zinc trasporter that confers resistance to zinc. EMBO J. 14, 689-649.

Palmiter, R.D. (1987) Molecular biology of metallothionein gene expression. Exs. 63-80.

Palmiter, R.D. (1994) Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proc. Natl. Acad. Sci. USA 91, 1219-1223.

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

Pawlowski, T.J., and Staerz, U.D. (1994) Thymic education- T cells do it for themselves. Immun. Today 15, 205-209.

Perkins, D.J. (1964) Zinc binding to poly-L-glutamic acid and human serum albumin. Biochem. Biophys. Acta. 86, 635-636.

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

Picker, L.J., and Butcher, E.C. (1992) Physiological and molecular mechanisms of lymphocyte homing. Annu. Rev. Immunol. 10, 561-591.

Powell, S., Saltman, P., Uretzky, G., and Chevion, M. (1990) The effect of zinc on reperfusion arrhythmias in the isolated perfused rat heart. Free Radic. Biol. Med. 8, 33-46.

Powell, S.R., Hall, D., Aiuto, L., *et al.* (1994) Zinc improves postischemic recovery of isolated rat hearts through inhibition of oxidative stress. Am. J. Physiol. 266, H2497-H2507.

Powell, S.R., and Tortalani, A.J. (1992) Recent advances in the role of reactive oxygen intermediates in ischemic injury. J. Surg. Res. 53, 417-429.

Prasad, A.S., Rabbani, P., Abbasi, A., *et al.* (1978) Experimental zinc deficiency in humans. Ann. Intern. Med. 89, 483-490.

Prasad, A.S. (1985) Clinical, endocrinological and biochemical effects of zinc deficiency. In M.P. Cohen and P.P. Fodk (eds), *Special Topics in Endocrinology and Metabolism, Vol. 7*, Alan R. Liss, New York, pp 45-76.

Prasad, A.S. (1979) Clinical, biochemical and pharmacological role of zinc. Ann. Rev. Pharmacol. Toxicol. 20, 393-426.

Prasad, A.S. (1988) Clinical spectrum and diognostic aspects of human zinc deficiency. *Essential and Toxic Trace Elements in Human Health and Disease. Aspects of Zinc Deficiency*, Alan R. Liss, Inc., pp 3-53.

Prasad, A.S. (1995) Zinc: an overview. Nutrition 11, 93-99.

Prasad, A.S., Miale, A., Farid, Z., *et al.* (1963) Zinc metabolism in patients with the syndrome of iron deficiency anemia, hypogonadism, and dwarfism. J. Lab. Clin. Med. 61, 537-549.

Prasad, A.S., Halsted, J.A., and Nadimi, M. (1961) Syndrome of iron deficiency, anemia, hepatosplenomegaly, hypogonadism, dwarfism, and geophagia. Am. J. Med. 31, 532-546.

Prasad, A.S. (1991) Discovery of human zinc deficiency and studies in experimental human model. Am. J. Clin. Nutr. 53, 403-412.

Prasad, A.S. (1993) Acquired zinc deficiency and immune dysfunction in sickle cell anemia. In S. Cunningham-Rundles (ed), *Nutrient Modulation of the Imune Response*, Dekker, New York, pp 393-410.

Prasad, A.S., Meftah, S., Abdallah, J., *et al.* (1988) Serum thymulin in human deficiency. J. Clin. Invest. 82, 1202-1210.

Prasad, A.S., Fitzgerald, J.T., Hess, J.W., *et al.* (1993) Zinc deficiency in the elderly. Nutrition 9, 218-224.

Pratt, W.B., Scherrer, L.C., Hutchinson, K.A., and Dalman, F.C. (1992) A model of glucocorticoid receptor unfolding and stabilization by a heat shock protein. J. Steroid Biochem. Mol. Biol. 41, 223-229.

Pratt, W.B., and Scherrer, L.C. (1994) Heat shock proteins and the cytoplasmic nuclear trafficking of steroid receptors. In V.K. Moudgil (ed), *Steroid Hormone Receptors: Basic and Clinical Aspects*, Birkhäuser, Boston, pp 215-246.

Pratt, W.B., Sanchez, E., Bresnick, E.H., *et al.* (1989) Interaction of the glucocorticoid receptor with the Mr 90,000 heat shock protein: an evolving model of ligand-mediated receptor transformation and translocation. Cancer Res. 49, 2222s-2229s.

Pratt, W.B. (1987) Transformation of glucocorticoid and progesterone receptors to the DNA-binding state. J. Cell. Biochem. 35, 51-68.

Pratt, W.B. (1993) The role of heat shock proteins in regulating the function, folding, and trafficing of the glucocorticoid receptor. J. Biol. Chem. 268, 21455-21458.

Pratt, W.B. (1990) Interaction of hsp90 with steroid receptors: organizing some diverse observations and presenting the newest concepts. Mol. Cell Endocrinol. 74, C69-C76.

Pratt, W.B. (1992) Control of steroid receptor function and cytoplasmic nuclear transport by heat shock proteins. BioEssays 14, 841-848.

Pratt, W.B., Jolly, D.J., Pratt, D.V., *et al.* (1988) A region of the steroid binding domain determines formation of the non-DNA-binding of 9S glucocorticoid receptor complex. J. Biol. Chem. 263, 267-273.

Quarterman, J. (1974) The effects of zinc deficiency or excess on the adrenal glands and the thymus of the rat. In W.G. Hoekstra, J.W. Suttie, H.E. Ganther, and W. Mertz (eds), *Trace Element Metabolism in Animals*, University Park, Baltimore, MD, pp 742-748.

Quarterman, J. (1972) The effects of zinc deficiency on the activity of the adrenal glands. Proc. Nutr. Soc. 31, 74A-75A.

Quarterman, J., and Humphries, W.R. (1979) Effect of zinc-deficiency and zinc supplementation on adrenals, plasma steroids and thymus in rats. Life Sci. 24, 177-183.

Rabbani, P.I., Prasad, A.S., Tsai, R., *et al.* (1987) Dietary model for production of experimental zinc deficiency in man. Am. J. Clin. Nutr. 45, 1514-1525.

Raffaniello, R.D., and Wapnir, R.A. (1991) Zinc-induced metallothionein synthesis by Caco-2 cells. Biochem. Med. & Met. Biol. 45, 101-107.

Raffaniello, R.D., and Wapnir, R.A. (1989) Zinc uptake by isolated rat enterocytes: effect of low molecular weight ligands. Proc. Soc. Exp. Biol. Med. 192, 219-224.

Raffaniello, R.D., Lee, S-Y., Teichberg, S., and Wapnir, R.A. (1992) Distinct mechanisms of zinc uptake at the apical and basolateral membranes of Caco-2 cells. J. Cell. Physiol. 152, 356-361.

Rajaram, S., Carlson, S.E., Koo, W., and Braselton, W.E. (1995) Plasma mineral concentrations in preterm infants fed a nutrient-enriched formula after hospital discharge. J. Pediat. 126, 791-796.

Reed, J.C. (1994) Bcl-2 and the regulation of programmed cell death. J. Cell Biol. 124, 1-6.

Reeves, P.G., Nielsen, F.H., and Fahey, G.C. Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76Z rodent diet. J. Nutr. 123, 1939-1951.

Ren, Y., Silverstein, R.L., Allen, J., and Savill, J. (1995) CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. J. Exp. Med. 181, 1857-1862.

Reul, J.M., and de Kloet, E.R. (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505-21511.

Reul, J.M.H.M., van den Bosch, F.R., and de Kloet, E.R. (1987) Differential response of type I and type II corticosteroid receptors to changes in plasma steroid level and circadian rhythmicity. Neuroendocrinology 45, 407-412.

Revera, J., Brown, K.H., Santizo, M.C., *et al.* (1995) Effects of zinc supplementation on the growth of young Guatemalan children (abstract). FASEB J. 9, A164.

Rhodes, D., and Klug, A. (1993) Zinc fingers. Scientific Am. Feb., 56-65.

Richard, M.J., Arnold, J., Jurkovitz, C., *et al.* (1991) Trace elements and lipid peroxidation abnormalities in patients with chronic renal failure. Nephron 57, 10-15.

Richards, M.P., and Cousins, R.J. (1976) Metallothionein and its relationship to the metabolism of dietary zinc rats. J. Nutr. 106, 1591-1599.

Richards, M.P., and Cousins, R.J. (1975) Mammalian zinc homeostasis: requirement for RNA and metallothionein synthesis. Biochem. Biophys. Res. Comm. 64, 1215-1223.

Rinner, L., Schauenstein, K., Mangge, H., *et al,* (1992) Opposite effects of mild and severe stress on *in vitro* activation of rat peripheral blood lymphocytes. Brain Behav. Immun. 6, 130-140.

Ripa, S., and Ripa, R. (1995) Zinc in immune function. Minerva Med. 86, 315-318.

Robinson, P.A., Langley, M.S., and Hammond, G.L. (1985) Identification and characterization of a human corticosteroid binding globulin variant with a reduced affinity for cortisol. J. Endocrinol. 104, 269-277.

Roes, J., and Rajewsky, K. (1993) Immunoglobulin D (IgD)-deficient mice reveal an auxiliary receptor function for IgD in antigen-mediated recruitment of B cells. J. Exp. Med. 177, 45-55.

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

Rolink, A., Karasuyama, H., Haassner, D., *et al.* (1994) Two pathways of B-lymphocyte developement in mouse bone marrow and the roles of surrogate L chain in this developement. Immunol. Rev. 137, 185-201.

Rolink, A., Kudo, A., Karasuyama, H., *et al.* (1991) Long-term proliferating early pre b-cell lines and clones with the potential to develope to surface Ig-positive, mitogen reactive B-cell *in vito* and *in vivo*. EMBO J. 10, 327-336.

Rolink, A., and Melchers, F. (1993) Generation and regeneration of cells of the B-lymphocyte lineage. Curr. Opin. Immunol. 5, 207-217.

Rosenstein, Y., Park, J.K., Hahn, W.C., *et al.* (1991) CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. Nature 354, 233-235.

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

Rothman, J.E. (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59, 591-601.

Rothstein, D.M., da Silva, A., Sugita, K., *et al.* (1993) Human CD4/CD45RA$^+$ and CD4/CD45RA$^-$ T cell subsets express CD4-p561ck complexes, CD4-associated lipid kinases, TCR/CD3-p59fyn complexes, and share similar tyrosine kinase substrates. Int. Immunol. 5, 409-418.

Rudney, H., and Sexton, R.C. (1986) Regulation of cholesterol biosynthesis. Ann. Rev. Nutr. 6, 245-272.

Saha, A.P., Hadden, E.M., and Hadden, J.W. (1995) Zinc induces thymulin secretion from human thymic epithelial cells *in vitro* and augments splenocyte and thymocyte responses *in vivo*. Int. J. Immunopharmacol. 17, 729-733.

Sakaguchi, N., Kashiwamura, S.I., Kimoto, M., *et al.* (1988) B lymphocyte-lineage-restricted expression of mb-1, a gene with CD3-like structural properties. EMBO J. 7, 3457-3464.

Sakai, R.R., and Epstein, A.N. (1990) Dependence of adrenalectomy-induced sodium appetite on the action of angiotensin II in the brain of the rat. Behav. Neurosci. 104, 167-176.

Sammar, M., Aigner, S., Hubbe, M., *et al.* (1994) Heat-stable antigen (CD24) as ligand for mouse P-selectin. Int. Immunol. 6, 1027-1036.

Sanchez, E.R., Hirst, M., Scherrer, L.C., *et al.* (1990a) Hormone-free mouse glucocorticoid receptors overexpressed in chinese hamster ovary cells are localized to the nucleus and are associated with both hsp70 and hsp90. J. Biol Chem. 265, 20123-20130.

Sanchez, E.R., Faber, L.E., Henzel, W.J., and Pratt, W.B. (1990b) The 56-59 kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exist in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. Biochemistry 29, 5145-5152.

Sanchez, E.R., Hausley, P.R., and Pratt, W.B. (1986) The molybdate-stabilized glucocorticoid binding complex of L-cells contains a 98-100 KDa steroid binding phosphoprotein and or 90 KDa non-steroid-binding phosphoprotein that is part of the murine heat-shock complex. J. Steroid Biochem. 24, 9-18.

Sandström, B., and Lönnerdal, B. (1989) Promoters and antagonists of zinc absorption. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 57-78.

Savill, J.S. (1995) The innate immune system: Recognition of apoptotic cells. In C.D. Gregory (ed), *Apoptosis and the Immune Reponse*, Willy-Liss, Inc., New York, pp. 152-171.

Savill, J.S., Wyllie, A.H., Henson, J.E., *et al.* (1989a) Macrophage phagacytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to recognition by macrophages. J. Clin. Invest. 83, 865-895.

Savill, J.S., Henson, P.M., and Haslett, C. (1989b) Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge sensitive" recognition mechanism. J. Clin. Invest. 84, 1518-1527.

Schroeder, J.J., and Cousins, R.J. (1990) Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proc. Natl. Acad. USA 87, 3137-3141.

Schroeder, J.J., and Cousins, R.J. (1991) Maintenance of zinc-dependent hepatic functions in rat hepatocytes cultured in medium without added zinc. J. Nutr. 121, 844-853.

Schwabe, J.W., and Klug, A. (1994) Zinc mining for protein domains. Nat. Struct. Biol. 1, 345-349.

Schwartzman, R.A., and Cidlowski, J.A. (1993) Mechanism of tissue-specific induction of internucleosomal deoxyribonucleic acid cleavage activity and apoptosis by glucocorticoids. Endocrinology 133, 591-599.

Schwarz, M.A. (1994) Cytoplasmic metallothionein overexpression protects NIH 3T3 cells from tert-butyl hydroperoxide toxicity. J. Biol. Chem. 269, 15238-15243.

Scott, D.W. (1995) Apoptosis in immature B-lymphocytes. In C.D. Gregory (ed), *Apoptosis and the Immune Response*, Willey-Liss, New York, pp 187-215.

Scrocchi, L.A., Hearn, S.A., Han, V.K.M., and Hammond, G.L. (1993a) Corticosteroid-binding globulin biosynthesis in the mouse liver and kidney during postnatal development. Endocrinology 132, 910-916.

Scrocchi, L.A., Orava, M., Smith, C.L., *et al.* (1993b) Spatial and temporal distribution of carticosteroid-binding and its messenger ribonucleic acid in embryonic and fetal mice. Endocrinology 132, 903-909.

Seal, C.J., and Heaton, F.W. (1987) Zinc transfer among proteins in rat duodenum mucosa. Ann. Nutr. Metab. 31, 55-60.

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

Selye, H. (1956) The stress of life. McGraw-Hill, New York.

Sentman, C.L., Shutter, J.R., Hockenbery, D., *et al.* (1991) Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67, 879-888.

Seralini, G.E., Berube, D., Gagne, R., and Hammond, G.L. (1990) The human corticosteroid binding globulin gene is located on chromosome 14q31-q32.1 near two other serine protease inhibitor genes. Hum. Genet. 86, 73-75.

Shambaugh, G.E. Jr. (1989) Zinc: the neglected nutrient. Am. J. Otol. 10, 156-160.

Sherman, A.R. (1992) Zinc, copper, and iron nutriture and immunity. J. Nutr. 122, 604-609.

Shi, Y., Bissonnettem R.P., Parfrey, N., *et al.* (1991) *In vivo* administration of antibodies to the CD3 T-cell receptor complex induces cell death (apoptosis) in immature thymocytes. J. Immunol. 146, 3340-3346.

Shi, H.N., Scott, M.E., Koski, K.G., *et al.* (1995) Energy restriction and severe zinc deficiency influence growth, survival and reproduction of *Heligmosomoides polygyrus* (Nematoda) during primary and challenge infections of mice. Parasitology 110, 599-609.

Shi, S., and Thomas, J.O. (1992) The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol. Cell. Biol. 12, 2186-2192.

Shippee, R.L., Koppenheffer, T., Watiwat, S.R., *et al.* (1992) The interaction of burn injury and zinc nutriture as assessed by the humoral response to sheep red blood cells in a burn rat model. Burns 1, 45-48.

Shortman, K., Egerton, M., Spangrude, G.J., and Scollay, R. (1990) The generation and fate of thymocytes. Semin. Immunol. 1, 3-12.

Siiteri, P.K., Murai, J.T., Hammond, G.L., *et al.* (1982) The serum transport of steroid hormones. Rec. Prog. Horm. Res. 38, 457-510.

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

Slaunwhite, W.R. Jr., Lockie, G.N., Back, N., and Sandberg, A.A. (1962) Inactivity *in vivo* of transcortin-bound cortisol. Science 135, 1062-1063.

Sloviter, R.S., Valiquette, G., Abrams, G.M., *et al.* Selective loss of hippocampal granaule cells in the mature rat brain after adrenalectomy. Science 243, 535-538.

Smith, H.F., Latham, M.C., Azaburke, J.A., *et al.* (1981) Blood plasma levels of cortisol, insulin, growth hormone, and somatomedin in children with marasmus, kwashiorkor and intermediate forms of protein-energy malnutrition. Proc. Soc. Exp. Biol. Med. 167, 607-611.

Smith, C.A., Williams, G.T., Kingston, R., *et al.*(1989) Antibodies to CD3 T-cell receptor complex induce death by apoptosis in immature T-cells in thymic culture. Nature 337, 181-84.

Smith, D.F., and Toft, D.O. (1993) Steroid receptors and their associated proteins. Mol. Endocrinal. 7, 4-11.

Sobocinski, P.Z., Canterbury, W.J., Mapes, C.A., and Dinterman, R.E. (1978) Involvement of hepatic metallothionein in hypozincemia associated with bacterial infection. Am. J. Physiol. 234, E399-406.

Solomons, N.W., and Cousins, R.J. (1984) Zinc. In N.W. Solomons and I.H. Rosenberg (eds), *Absorption and malabsorption of mineral nutrients,* Alan R. Liss, New York, pp 125-197.

Spencer, R.L., Miller, A.H., Moday, H., *et al.* (1993) Diurnal differences in basal and acute stress levels of type I and type II adrenal steroid receptor activation in neural and immune tissues. Endocrinology 133, 1941-1950.

Spencer, R.L., Miller, A.H., Stein, M., and McEwen, B.S. (1991) Corticosterone regulation of type I and type II adrenal steroid receptors in brain pituitary and immune tissue. Brain Res. 549, 236-246.

Sprent, J. (1989) T lymphocytes and the thymus. Fund. Immun., 2nd edition, 69-93.

Sprent, J., Lo, D., Gao, E.-K, and Ron, Y. (1988) T cell selection in the thymus. Immunol. Rev. 101, 173-190.

Sprent, J., and Webb, S.R. (1987) Function and specificity of T cell subsets in the mouse. Adv. Immunol. 41, 39-133.

Steel, L., and Cousins, R.J. (1985) Kinetics of zinc absorption by luminally and vascularly perfused rat intestine. Am. J. Physiol. 248, G46-G53.

Steel, R.G.D. and Torrie, J.H. (1980) *Principles and Procedures of Statistics: A Biometrical Approach*, McGraw Hill, New York, pp 185-186 and pp 544-545.

Strasser, A., Harris, A.W., Jacks, T., and Cory, S. (1994) DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell 79, 329-339.

Sun, X.M., Dinsdale, D., Snowden, R.T., *et al.* (1992) Characterization of apoptosis in thymocytes isolated from dexamethasone-treated rats. Biochem. Pharmacol. 44, 2131-2137.

Sunderman, F.W., Jr., (1995) Influence of Zinc on apoptosis. Ann. Clin. Lab. Science 25, 134-142.

Sutanto, W., and deKloet, E.R. (1987) Species-specificity of corticosteroid receptors in hamster and rat brains. Endocrinology 121, 1405-1411.

Symington, F.W., and Hakomori, S. (1984) Hematopoietic subpopulations express cross reactive, lineage-specific molecules detected by monoclonal antibody. Mol. Immunol. 21, 507-514.

Tacnet, R., Watkins, D.W., and Ripoche, P. (1990) Studies of zinc transport into brush-border membrane vesicles isolated from pig small intestine. Biochim. Biophys. Acta. 1024, 323-330.

Tai, P.K.K., Albers, M.W., Chang, H., *et al.* (1992) Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. Science 256, 1315-1318.

Tarlinton, D. (1994) B-cell differentiation in the bone marrow and the periphery. Immunol. Rev. 137, 203-229.

Taylor, C.G., Bettger, W.J., and Bray, T.M. (1988) Effects of dietary zinc or copper deficiency on the primary free radical defense system in rats. J. Nutr. 118, 613-621.

Telford, W.G., and Fraker, P.J. (1995) Preferential induction of apoptosis in mouse $CD4^+CD8^+$ $\alpha\beta TCR^{lo}CD3\epsilon^{lo}$ thymocytes by zinc. J. Cell. Physiol. 164, 259-270.

Telford, W.G., King, L.E., and Fraker, P.J. (1994) Rapid quantitation of apoptosis in pure and heterogeneous cell populations using flow cytometry. J. Immunol. Meth. 172, 1-16.

Telford, W., King, L., and Fraker, P.J. (1991) Evaluation of glucocorticoid induced DNA fragmentation in mouse thymocytes by flow cytometry. Cell Prolif. 24, 447-459.

Thiele, D.J. (1992) Metal-regulated transcription in eukaryotes. Nucleic Acid Res. 20, 1183-1191.

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

Thompson, E.B. (1994) Apoptosis and steroid hormones. Molec. Endoc. 8, 665-673.

Thornalley, P. J., and Vasak, M. (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanisms of its reaction with superoxide and hydroxyl radicals. Biochim. Acta 827, 36-44.

Todd, W.R., Elvehjen, C.A., and Hart, E.B. (1934) Zinc in the nutrition of the rat. Am. J. Physiol. 107, 146-156.

Tough, D.F., and Sprent, J. (1994) Turnover of naive- and memory-phenotype T-cells. J. Exp. Med. 179, 1127-1135.

Treves, S., Trentini, P.L., Ascanelli, M., *et al.* (1994) Apoptosis is dependent on intracellular zinc and independent of intracellular calcium in lymphocytes. Exp. Cell. Res. 211, 339-343.

Tsai, S., Bartelmez, S., Sitnicka, E., and Collins, S. (1994) Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. Genes Dev. 8, 2831-2841.

Vacchio, M.S., Papadopoulos, V., and Ashwell, J.D. (1994) Steroid production in the thymus: implications for thymocytes selection. J. Exp. Med. 179, 1835-1846.

Vallee, B.L., and Falchuk, K.H. (1993) The biochemical basis of zinc physiology. Physiol. Rev. 73, 79-118.

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

Vallee, B.L., and Auld, D.S. (1993) New Perspective on Zinc Biochemistry: Cocatalytic Sites in Multi-Zinc Enzymes. Amer. Chem. Soc. 23, 6494-6500.

Vallee, B.L., and Galdes, A. (1984) The metallobiochemistry of zinc enzymes. Adv. Enzymol. Relat. Areas Mol. Biol. 56, 283-430.

Vallee, B.L., and Auld, D.S. (1990) Zinc corrdination, function, and structure of zinc enzymes and other proteins. Biochemistry 19, 5647-5659.

van Ewijk, W. (1991) T cell differentiation is influenced by thymic microenvironments. Annu. Rev. Immunol. 9, 591-615.

Van Wouwe, J. P. (1989) Clinical and laboratory diagnosis of *Acrodermatitis enteropathica*. Eur. J. Pediatr. 149, 2-8.

Veis, D.J., Sentman, C.L., Bach, E.A., and Korsmeyer, S.J. (1993) Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. J. Immunol. 151, 2546-2554.

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

Voetberg, B.J., Garvy, B.A., Mayer, H.K., *et al.* (1994) Apoptosis accompanies a change in the phenotypic distribution and functional capacity of murine bone marrow B-cells chronically exposed to prednisalone. Clin. Immunol. Immunopath. 71, 190-198.

von Boehmer, H. (1992) Thymic selection: a matter of life and death. Immunol. Today 13, 454-458.

von Boehmer, H., and Kisielow, P. (1990) Self-nonself discrimination by T cells. Science 248, 1369-1373.

Vruwink, K.G., Hurley, L.S., Gershwin, M.E., and Keen, C.L. (1988) Gestational zinc deficiency amplifies the regulation of metallothionein induction in adult mice. Proc Soc. Exp. Biol. Med. 188, 30-34.

Walker, N.I., Harmon, B.V., Gobe, G.C., and Kerr, J.F. (1988) Patterns of cell death. Methods Achiev. Exp. Pathol. 13, 18-54.

Walsh, C.T., Sandstead, H.H., Prasad, A.S., *et al.* (1994) Zinc: health effects and research priorities for the 1990s. Environ. Health Perspect. 102, 5-46.

Watanabe, T., Arakawa, T., Fukuda, T., *et al.* (1995) Zinc deficiency delays gastric ulcer healing in rats. Dig. Dis. Sci. 40, 1340-1344.

Webb, M. (1986) Role of metallothionein in cadmium metabolism. Handb. Exp. Pharmacol. 80, 281-337.

Weiser, J.N., Do, Y.S., and Feldman, D. (1979) Synthesis and secretion of corticosteroid-binding globulin by rat liver. J. Clin. Invest. 63, 461-467.

Weissman, K., Hoe, S., Knudsen, L., and Sorensen, S.S. (1979) Zinc absorption in patients suffering from *Acrodermatitis enteropathica* and in normal adults assessed by whole-body counting technique. Br. J. Dermatol. 1010, 573-579.

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

Welch, P.A., Burrows, P.D., Namen, A., *et al.* (1990) Bone marrow stromal cells and interleukin-7 induce coordinate expression of the BP-1/6C3 antigen and pre-B cell growth. Inter. Immunol. 2, 697-705.

Wenger, R.H., Rochelle, J.M., Seldin, M.F., *et al.* (1993) The heat-stable antigen (mouse CD24) gene is differentially regulated but has a housekeeping promoter. J. Biol. Chem. 268, 23345-23352.

Wenger, R.H., Ayane, M., Bose, R., *et al.* (1991) The genes for a mouse hemopoietic differentiation marker called the heat-stable antigen. Eur. J. Immunol. 21, 1039-1046.

Werthamer, S., Govindaraj, S., and Amaral, L. (1976) Placenta, trascortin, and localized immunoresponse. J. Clin. Invest. 57, 1000-1008.

Werthamer, S., Samuels, A.J., and Amnral, L. (1973) Identification and partial purification of "transcortin" like protein within human lymphocytes. J. Biochem. 248, 6398-6407.

Whitehead, R.G., Coward, W.A., and Lunn, P.G. (1973) Serum-albumin concentration and the onset of Kwashiorkor. Lancet I, 63-66.

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

Whitlock, C.A., Tidmarsh, G.F., Muller-Sieburg, C., and 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.

Wilder, R.L. (1995) Neuroendocrine-immune system interactions and autoimmunity. Annu. Rev. Immunol. 13, 307-338.

Williams, G.T. (1994) Apoptosis in the immune system. J. Pathology. 173, 1-4.

Williams, R.J.P. (1989) An introduction to the biochemistry of zinc. In C.F. Mills (ed), *Human Nutrition Reviews. Zinc in Human Biology*, Springer-Verlag, London, pp 15-31.

Wilson, R.L. (1989) Zinc and iron in free radical pathology and cellular control. In C.F. Mills (ed) *Human Nutrition Reviews, Zinc in Human Biology*, Springer-Verlag, London, pp 147-172.

Wirth, J.J., Fraker, P.J., and Kierszenbaum, F. (1989) Zinc requirement for macrophage function: effect of zinc deficiency on uptake and killing of a protozoan parasite. Immunology 68, 114-119.

Woodward, B. (1991) Zinc, a pharmacologically potent essential nutrient: focus on immunity. Can. Med. Assoc. J. 145, 1469.

Wu, Q., Li, L., Cooper, M., *et al.* (1991) Aminopeptidase A activity of the murine B-lymphocyte differentiation antigen BP-1/6C3. Proc. Natl. Acad. Sci. USA 88, 676-680.

Wu, Q., Tidmarsh, G.F., Welch, P.A., *et al.* (1989) The early B lineage antigen BP-1 and the transformation-associated antigen 6C3 are on the same molecule. J. Immunol. 143, 3303-3308.

Wyllie, A.H. (1987) Apoptosis: cell death under homeostatic control. Arch. Toxicol. 11, 3-10.

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. (1980) Cell death: the significance of apoptosis. Int. Rev. Cytol. 68, 251-305.

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

Yamanashi, Y., Kakuichi, T., Mizuguchi, J., *et al.* (1991) Association of B-cell antigen receptor with protein tyrosine kinase Lyn. Science 251, 192-194.

Yonemura, S., Nagafuchi, A, Sato, N., and Tsukita, S. (1993) Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. J. Cell. Biol. 120, 437-449.

Yu, D.T.Y., Clements, P.J., Paulus, *et al.* (1974) Human lymphocyte subpopulations. J. Clin. Invest. 53, 565-571.

Zeng, B., Qian, Y., Zheng, D., *et al.* (1991) Change of T lymphocyte subsets in peripheral blood of children with malnutrition and zinc deficiency. Hua. Hsi. I. Ko. Ta. Hsueh. Hsueh. Pao. 22 (3), 337-339.