DIETARY ZINC DEFICIENCY AND PROTEIN-ENERGY MALNUTRITION DECREASE IN VITRO MURINE T-LYMPHOCYTE CELL CYCLE PROGRESSION

by Pamela J. Bossuyt

A thesis submitted to the Department of Foods and Nutrition
in partial fulfillment of the requirements
for the degree of Master of Science

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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PAMELA J. BOSSUYT

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Dietary Zinc Deficiency and Protein-Energy Malnutrition Decrease in vitro Murine T-lymphocyte Cell Cycle Progression

P.J. Bossuyt, MSc Thesis, Department of Foods and Nutrition

Both zinc (Zn) deficiency and protein-energy malnutrition (PEM) have been shown to have adverse effects on immune function, including a decrease in the number of T-lymphocytes and a decreased proliferative response of T-lymphocytes when stimulated in culture. Flow cytometry is a method that enables the identification of T-lymphocytes subpopulations by their surface receptors [ie. CD4+ (T-helper cells) and CD8+ (T-cytotoxic/suppressor cells)] and the identification of cells in each phase of the cell cycle. The objective of this thesis was to investigate the effects of dietary zinc deficiency and PEM, alone and in combination, compared to a control group fed a nutritionally adequate diet, on murine splenic T-lymphocyte cell cycle progression and T-lymphocyte subpopulations, immunological parameters (spleen weight and the number of splenocytes/spleen) and zinc status.

In two separate experiments, 35 female C56BL/6 adult mice were randomly assigned to 5 treatment groups [Zn deficient & low protein (ZnDF&LP), Zn deficient (ZnDF), low protein (LP), energy restricted (ER), and control (CTRL)] and fed for 4 weeks. Body weights and spleen weights of all four deficient groups were significantly lower than CTRL in both experiments. However, only the LP group had splenocyte counts significantly lower than CTRL. ZnDF&LP, ZnDF and LP groups had significantly lower serum Zn concentrations than CTRL. In Experiment 1, it was shown that the CD4+/CD8+ ratio of T-lymphocytes was unaltered by dietary deficiencies of zinc and

protein. However, the ZnDF&LP group had a decreased percentage of CD4+ cells (23.3%) compared to the CTRL, and there was evidence that all four deficient groups may have increased numbers of immature "double-negative" T-lymphocytes compared to the CTRL group. Cell cycle analysis in Experiment 2 revealed that all deficient groups had a lower percentage of ConA-stimulated cells in S phase (15.8%-18.9%) in the presence of 2-mercaptoethanol compared to CTRL (25.7%). ZnDF&LP, ZnDF and LP groups had a higher percentage of cells in the resting (G_0) phase of the cell cycle (78.8%-80.0%) compared to CTRL (70.4%).

In conclusion, it appears that dietary deficiencies of Zn and protein, either alone or in combination, decrease body weight, spleen weight and serum Zn concentrations. These dietary deficiencies also affect the progression of cells through the cell cycle, by decreasing the percentage of cells in S phase and increasing the percentage of cells in the resting phase. Further research is needed to determine the specific molecular mechanisms by which Zn deficiency inhibits cell cycle progression into S phase.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

μg	microgram
μM	micromolar
2-ME	2-mercaptoethanol
AE	Acrodermatitis enteropathica
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
ConA	ConcanavalinA
CTRL	Control
ER	Energy restricted
FITC	fluorescein isothiocyanate
g	gram
GSH	Glutathione
GSSG	Disulfide form of glutathione
IL-2	interleukin-2
LP	Low protein
PBS	Phosphate buffered saline
PE	phycoerythrin
PEM	Protein-energy malnutrition
PI	Propidium iodide
ppm	parts per million
SRBC	Sheep red blood cells
Zn	Zinc
ZnDF	Zinc deficient
ZnDF&LP	Zinc deficient and low protein

I. LITERATURE REVIEW

Despite the fact that the functional roles of zinc in biochemistry are well known, the molecular mechanism of zinc deficiency pathology has not been elucidated. This thesis will address the effects of protein-energy malnutrition (PEM) and zinc deficiency on the cell-mediated immune system.

Nutrition and Immune Function

Despite the fact that improper nutrition has likely been a causative factor in decreased immunocompetence for centuries, the study of nutrition and its relationship to the immune system has only become an area of scientific study over the past few decades. The effect of malnutrition on the immune system is especially apparent in many third world and developing countries, where widespread malnutrition and infection are an every day concern, with one condition aggravating the other. In Latin America, nutritional deficiency was an associated cause in 60.9% of deaths from infectious diseases in children less than 5 years of age, as compared with only 32.7% of deaths from all other causes (Heywood & Marks, 1993).

The immune system is comprised of a variety of highly specialized cells intricately coordinated to protect the body against invading pathogens. There are two classifications of specific immune responses: 1) humoral immunity and 2) cell-mediated immunity. The classifications are based on the components of the immune system that mediate the response. The components of the humoral immune system that mediate immune response are antibodies, while the cell-mediated immune response involves cells referred to as T-lymphocytes, which function in antigen recognition. T-lymphocytes

originate in the bone marrow and then migrate to and mature in the thymus. T-lymphocytes are further subdivided into two functionally distinct populations, helper T-cells (CD4+) and cytotoxic/suppressor T-cells (CD8+). The first exposure of the immune system to a foreign antigen results in a relatively small primary response. When a T-lymphocyte recognizes a foreign antigen, the cell becomes activated, initiating a cascade of intracellular biochemical events within the T-cell that result in cellular immune response. This cascade of events is referred to as the T-lymphocyte signal transduction pathway. The responses of the immune system to second and subsequent exposures to the same antigen are called the secondary immune response. Generally, the secondary immune response is more rapid and larger than the primary response (Abbas et al, 1991).

Cells involved in the cell-mediated immune system have a high rate of turnover and their function depends on metabolic pathways that employ various nutrients as cofactors (Chandra, 1991). Both of these factors make lymphoid tissues and cells very vulnerable to malnutrition. Damaged tissues or immune cells will decrease the function of the body's immune system, and thus the body is less able to mount an effective defense to infection and disease. This decreased resistance results in increased opportunity for infection to occur, which in turn can worsen an already compromised nutritional state. This is the vicious cycle of malnutrition and infection. Compromised immune function can result from either generalized malnutrition or from a deficiency of a particular macronutrient, or micronutrient.

Zinc Deficiency

i) Human dietary zinc deficiency

Dietary zinc deficiency in humans was first recognized in 1963, when Iranian men presenting with iron deficiency anemia, hypogonadism and dwarfism were diagnosed as zinc deficient (Prasad et al, 1963). Since that time, zinc deficiency has become a concern for many subpopulations worldwide. Results from the Second National Health and Nutrition Examination Survey (NHANES II), which took place in the United States from 1976-1980, showed that women and the elderly had low zinc intakes primarily due to low energy intakes (Mares-Perlman et al, 1995). Sandstead (1995) also reported that premenopausal women in the United States are at risk of zinc deficiency due to the combination of innappropriate food selection (ie. low intake of meat and dairy products) and low energy intakes. Epidemiological studies in the United States have associated low plasma zinc levels with abnormal pregnancy outcomes and controlled intervention trials showed that zinc repletion improved pregnancy outcomes (Sandstead, 1995). Infants and children are also at risk of becoming zinc deficient due to their high requirement of zinc for growth (Gibson, 1989). A study in Canada found the existence of a growth-limiting zinc deficiency syndrome in 5-7 year old Southern Ontario boys with low height percentile (Gibson et al, 1989).

ii) Primary and secondary zinc deficiency

Although this thesis will focus on dietary zinc deficiency, it is important to be aware of other situations in which zinc deficiency may be a concern. Zinc deficiency due to inadequate dietary intake is called primary zinc deficiency, while zinc deficiency that occurs as a result of other factors is referred to as secondary zinc deficiency (Cunnane,

1988). One cause of secondary zinc deficiency is decreased zinc absorption, which may be due to a variety of factors, including: 1) gastro-intestinal dysfunction as a result of intestinal mucosal damage or malabsorption syndromes; 2) the absence of appropriate ligands which occurs in diseases such as acrodermatitis enteropathica or cystic fibrosis; and 3) dietary factors, such as phytate, calcium and alcohol, which bind zinc, limiting its absorption. An increased requirement for zinc or an increased utilization of zinc, which occurs in cases of surgery, burns, pregnancy, chronic infection or inflammation may also cause secondary zinc deficiency. A third factor that may cause secondary zinc deficiency is an increase in the excretion of zinc due to alcohol abuse, renal disease or sickle-cell disease (Cunnane, 1988). However, regardless of the cause of the deficiency, both primary and secondary zinc deficiency are characterized by the same clinical deficiency signs.

iii) Zinc deficiency signs

There is no remarkable or distinguishing feature of zinc deficiency and the signs could be attributed to a variety of causes. The general nature of zinc deficiency signs makes zinc deficiency difficult to diagnose. Due to zinc's role in cell proliferation, tissues with a rapid turnover, such as those involved with cell-mediated immunity, the intestinal mucosa and the skin, are particularly vulnerable to zinc deficiency (Aggett & Comerford, 1995). The degree of zinc deficiency, whether it be mild, moderate or severe, will determine the severity of the signs. Some features of zinc deficiency include: anorexia, weight loss, failure to thrive in infants and children, dermatitis, delayed puberty and hypogonadism, and increased susceptibility to infection. The increased susceptibility to infection is due to the fact that zinc deficiency results in an impaired immune system

(Aggett & Comerford, 1995), which will be discussed in more detail in the section on Zinc Deficiency and Immune Function.

iv) Zinc body pools

It is important that adults regularly consume a diet with adequate amounts of zinc because humans do not have the ability to readily mobilize zinc stores. The lack of these available stores, coupled with relatively high obligatory endogenous losses of zinc, requires humans to consume adequate amounts of zinc on a regular basis in order to maintain zinc plasma levels (Keen & Gershwin, 1990). The mechanism of zinc homeostasis is not well understood, however, it has been shown in humans that dietary zinc deficiency can cause a reduction in zinc from the body zinc pools (Miller et al, 1994). Since tissues conserve zinc, it has been suggested that only a small cellular pool of zinc is exchangeable (Cousins, 1996).

Two indicators of zinc status are serum or plasma zinc, representing short term zinc status, and femur zinc, a more long term indicator of zinc status. These two parameters are relevant to studies regarding immune function, since the precursors of T-lymphocytes originate in the bone marrow and then circulate to the plasma. Despite the fact that plasma zinc represents less than 1% of the total body content of zinc, it serves as the source of zinc that is accessible to all cells, but is rapidly depleted if zinc deficiency occurs (Vallee & Falchuk, 1993). Over 95% of zinc in plasma is carried by albumin and α_2-macroglobulin, therefore, it has been suggested that changes in the levels of proteins in the plasma may be related to changes in plasma zinc values, particularly in regards to albumin (Cunnane, 1996). However, Lepage (1997) did not find an association between serum zinc concentrations and serum albumin.

Bone has the highest zinc content in the body, representing 30% of total body zinc, and is fairly resistant to large variations in dietary zinc (Vallee & Falchuk, 1993). It has been suggested that two pools of zinc exist in bone, one which turns over rapidly and the other which turns over slowly. In times of zinc deficiency, zinc can be removed from the rapidly turning-over pool, however utilizing zinc from the slowly turning-over zinc pool results in bone loss (Zhou et al, 1993).

Functional Roles of Zinc

Although zinc is a trace element and the body's requirement for zinc is low in comparison to many other dietary components, it's role in the body is essential. Zinc is the most abundant intracellular trace element. It is a small ion that acts as a strong Lewis acid, causing it to bind strongly to thiolate and amine electron donors (Cousins, 1996). Zinc modulates the activity of approximately 300 enzymes and is essential for DNA synthesis, cell proliferation and gene expression (Prasad, 1995).

Zinc metalloenzymes are recognized in all classes of enzymes and catalyze approximately 50 important biochemical reactions. The functions of zinc in metalloenzymes include catalytic, structural, regulatory and noncatalytic. The level of zinc in cells may control the physiological processes through the formation and/or regulation of activity of zinc-dependent enzymes. Zinc exhibits fast ligand exchange which is important in its catalytic role in metalloenzymes (Cousins, 1996). Studies have shown that in zinc deficient rats, the activities of a few zinc-dependent enzymes, particularly thymidine kinase and alkaline phosphatase, were decreased when compared to their pair-fed controls (Prasad, 1996). In addition, relative amounts of thymidine

kinase mRNA are decreased when zinc deficiency is induced in cell lines, owing to a decreased translation of the gene (Prasad, 1996). However, there are also many zinc-containing metalloenzymes (ie. DNA polymerase, RNA polymerase) which do not seem to be directly affected by zinc deficiency, as the activity of these enzymes is decreased by other physiological conditions as well (ie. feed deprivation, stress, infection) (Bettger & O'Dell, 1993).

Zinc plays an integral role in gene expression, as it is a cofactor for a variety of metalloenzymes involved in nucleic acid metabolism. The role of zinc deficiency in gene expression has only been established recently in *Euglena gracilis* (Prasad, 1995). Zinc deficiency was shown to affect growth morphology, cell cycle and mitosis, suggesting that zinc plays a role in gene expression (Prasad, 1995). Zinc may also be involved in gene expression as a structural component of transcription factors. Transcription factors are regulatory proteins that are essential to the expression and regulation of genes because they act by binding to the recognition sequence of the appropriate gene. Without transcription factor binding, gene expression will not occur. A type of transcription factor that incorporates zinc as a structural part of the protein is called a zinc finger. (See section on Zinc Finger Proteins for more details). Studies have shown that the region of the transcription factor containing the zinc-binding domains is essential for DNA binding (Prasad, 1995).

Zinc also plays a critical physiological role in the structure and function of biomembranes, which may also contribute to the pathology of zinc deficiency (Bettger and O'Dell, 1993). Cellular structures that are in physical contact with the extracellular zinc pool will be the first to lose zinc in dietary zinc deficiency. The loss of zinc from

specific proteins in cell plasma membranes leads to altered membrane structure and function, which may in part account for some of the biochemical abnormalities seen in zinc deficiency.

Zinc Deficiency and Immune Function

During the past few decades, considerable knowledge has been gained regarding the role of zinc in cellular immunity, particularly in regards to T-lymphocyte function. Two naturally occurring zinc deficiency diseases, one in cattle and one in humans, provided initial evidence that adequate supplies of zinc are essential to the health and maintenance of the immune system. It was discovered that Dutch Fresian cattle with an A46 mutation inherit an inability to absorb zinc properly (reviewed in Hansen et al, 1982). These cattle appear normal at birth but begin to show common signs of zinc deficiency within the first few weeks of life. These cattle also become extremely susceptible to infection, which is the most common cause of death. However, supplementary zinc treatment will cause complete and rapid recovery of all zinc deficiency symptoms. Post-mortem examination of these calves revealed a severely impaired cell-mediated immune system. The thymus was severely decreased in size, which is an important indicator of decreased cellular immunity, because the thymus is the site of maturation of T-lymphocytes, which are integral to the cell-mediated immune system (reviewed in Hansen et al, 1982).

Acrodermatitis enteropathica (AE) is a zinc deficiency disease in humans analogous to the disease described above in cattle. Infants born with AE also appear normal at birth, but develop zinc deficiency signs within the first year of life. These

infants are also extremely susceptible to infection, unless zinc supplementation is provided. Abnormally small thymuses and a depleted number of T-lymphocytes occurs in infants with untreated AE, indicating that zinc deficiency affects the cell-mediated immune system (reviewed in Hansen et al, 1982).

In addition to these naturally occurring genetic zinc deficiency diseases the study of zinc deficiency in experimental animals, particularly mice and rats, has provided valuable information. Mice with zinc deficiency are more susceptible to pathogenic infection (Fraker et al, 1982; Salvin & Rabin, 1984) and have a decreased secondary immune response (DePasquale-Jardieu & Fraker, 1984). Results from studies with mice have also shown that dietary zinc deficiency adversely affects the lymphoid organs involved in the function of the cell-mediated immune system. Numerous studies have shown that thymus involution occurs in mice (Fraker et al, 1977; Fraker et al, 1978; Fernandes et al, 1979). Another lymphoid organ, the spleen, has also been shown to decrease in size (Fraker et al, 1977; Beach et al, 1980). The spleen is involved in cell-mediated immune function, as it serves as a storage organ for cells involved in cellular immunity, such as B- and T-lymphocytes and macrophages. Thus, many studies have focused on the effects of zinc deficiency on immune cells, particularly T-lymphocytes.

A study performed by Fraker and colleagues (1977) showed that young adult mice fed a zinc deficient diet for four weeks experienced rapid atrophy of the thymus. The function of T- and B-cells was assessed by their ability to produce antibodies in response to immunization with sheep red blood cells (SRBC). It was found that T-lymphocytes from zinc deficient animals had a decreased response to SRBC, but that B-cell response was not affected. It was also noted that the zinc deficient animals had a significantly

lower food intake, which is a well documented sign of prolonged zinc deficiency. In order to rule out the possible confounding factor of decreased energy intake on the parameters being assessed, they performed another study which included three dietary groups: a) a zinc supplemented group (50-60 μg/g Zn); b) a group fed the zinc supplemented diet, with their intake limited to that of the average daily amount consumed by the zinc deficient group (pair-fed group); and c) a zinc deficient group (0.5-0.6 μg/g Zn) (Luecke et al, 1978). The zinc deficient group experienced a pronounced loss in immune capacity, measured by their response to immunization with sheep red blood cells, compared to the pair-fed group and the control group. It was concluded that zinc deficiency per se, and not the decrease in energy intake was responsible for the adverse effects on the immune system, although the inanition did partially contribute to the loss in immunity (Luecke et al, 1978). Other studies have also found that zinc deficient rodents have compromised immune function as indicated by a decreased responsiveness of T-lymphocytes to SRBC (Fernandes et al, 1979; Chandra & Au, 1980).

Due to the association of decreased food intake with prolonged zinc deficiency, studies of zinc deficiency generally include either a pair-fed group or an energy restricted group to control for the effect of inanition. Pair-feeding involves determining the amount of diet consumed by the zinc deficient animals, and feeding the pair-fed group the same amount of the zinc adequate diet. This is especially difficult to manage when mice are studied, due to the fact that their powdered diet is easily spilled, making it hard to determine how much diet was spilled and how much was actually consumed. For this reason, an energy restricted group may be used instead of the pair-fed group. The energy restricted group is fed the zinc adequate diet in restricted amounts so that the weight of

these animals matches the average weight of the zinc deficient animals (ie. pair-weight group).

Many studies have shown that T-lymphocytes from zinc deficient rodents have a markedly reduced ability to proliferate when cultured in the presence of T-cell mitogens when compared to T-lymphocytes from rodents fed the control diet (Gross et al, 1979; Carlomagno & McMurray, 1983; Moulder & Steward, 1989). The decreased proliferative response indicates that the functionality of T-lymphocytes is affected by zinc deficiency. One study was found that contradicts this view. Cook-Mills & Fraker (1993) concluded that the decreased response of T-lymphocytes in zinc deficiency was only due to a decrease in the number of T-lymphocytes available to ilicit an immune response, and not due to a decrease in the functionality of remaining T-lymphocytes. Splenocytes from moderately or severely zinc deficient adult mice gave normal proliferative responses and generated adequate IL-2 activity when stimulated with ConcanavalinA (ConA), a T-cell mitogen. More details regarding the effect of zinc deficiency on T-lymphocyte proliferation will be presented in the section on Zinc and Cell Cycle.

It has been suggested that the decreased immune response, although not due to a decreased function of T-cells, may be due to an alteration in the proportion of the subpopulations of T-cells (ie. altered ratio of T-helper cells to T-suppressor cells). Significant changes in the proportion of lymphocyte subsets may lead to imbalances that affect both the response and regulation of the immune system. In one study (King & Fraker, 1991), flow cytometry was used to analyze the splenocytes of zinc deficient mice to determine whether the deficiency had altered the composition of T-cell subsets. The

two main subsets of T-lymphocytes are the T-helper cells (identified by the expression of a CD4+ receptor) and the T-cytotoxic/suppressor cells (identified by the expression of a CD8+ receptor). Although there was a significant decrease in total splenocyte numbers, there was no significant change in the composition of the splenic lymphocyte population subsets (King and Fraker, 1991). This supports the findings of previous studies, which found that zinc deficiency did not alter splenic T-lymphocyte subsets in rats (Bises et al, 1987; Dowd et al, 1986).

Many of these effects on the immune system can be completely reversed after a period of zinc repletion (Fraker et al, 1978), even when zinc deficiency occurs in young neonatal rats whose immune system is still developing (Zwickl & Fraker, 1980). This reversal has also been shown in children who have suffered from zinc deficiency as a result of malnutrition, as their immune function can be restored with adequate nutrition (Bhaskaram & Reddy, 1974).

Although the cause of the decreased lymphocyte counts in peripheral lymphoid organs is not known, two explanations are possible. One reason for the depressed number of lymphocytes may be simply due to a decrease in cell proliferation, thereby causing decreased production of lymphocytes. The other suggestion is that the decrease in T-cell numbers may be due to increased apoptosis, or increased destruction of T-lymphocytes.

Zinc Deficiency and Protein-energy Malnutrition

Another nutritional deficiency disease which compromises immune function is the classic malnutrition syndrome known as protein-energy malnutrition (PEM). In fact,

in many ways it is difficult to distinguish between zinc deficiency and PEM, as zinc deficiency generally occurs as a result of protein deficiency and the two deficiencies have common clinical features. Malnourished children have been shown to have decreased plasma zinc and other symptoms of zinc deficiency (Khalil et al, 1974; Golden & Golden, 1979; Atalay, 1989), which are resolved with appropriate levels of oral zinc supplementation (Hemalatha et al, 1993).

Part of the reason for this association is that meat is one of the main dietary sources of zinc. Therefore, when meat as a protein source is limited in the diet, not only will PEM result, but also zinc deficiency, unless other dietary sources of zinc are included in the diet. In many parts of the world where PEM occurs, such as developing countries, zinc deficiency is even more likely to occur in conjunction with PEM because of the high cereal consumption in these areas. Cereals contain phytate, which binds zinc, preventing its absorption. This compounds the problem, because, not only are these people consuming inadequate amounts of zinc, but the little zinc that they are consuming is not being absorbed.

Other characteristics of PEM that may contribute to decreased zinc status are diarrhea and steatorrhea which will reduce zinc absorption and increase zinc excretion (Anon, 1983).

A zinc deficient diet may also exacerbate conditions of PEM due to the fact that one of the classical symptoms of zinc deficiency is inanition, or decreased food intake. As a result, consumption of a zinc deficient diet for an extended period of time may result in the development of PEM, or may present a barrier to overcoming PEM.

Protein-energy Malnutrition and Immune Function

PEM affects the immune system very much the same way as zinc deficiency does. Compromised cellular immune function is frequently associated with PEM, particularly in regards to T-lymphocyte dependent functions. Atrophy of the thymus, decreased spleen weight and a significantly reduced number of T-lymphocytes were reported in PEM as early as 1971 (Smythe et al, 1971) and have been confirmed by numerous other researchers (Bhaskaram & Reddy, 1974; Chandra, 1991; Woodward et al, 1992). The responsiveness of T-lymphocytes to immunization with SRBC's is also diminished in PEM (Woodward et al, 1992; Woodward & Miller, 1991), as is the mitogenic response of T-lymphocytes in culture (Petro, 1985; Bhaskaram & Reddy, 1974).

Research regarding the effect of PEM on the composition of T-lymphocyte sub-populations is inconclusive. PEM is considered to induce a decrease in the numbers of CD4+ (T-helper) to CD8+ (T-cytotoxic/suppressor) T-lymphocytes in the blood, which is presumed to be related to a decrease in immune response. A low CD4+/CD8+ ratio commonly occurs in the blood of children with PEM (Chandra, 1983), however, the effect of PEM on the CD4+/CD8+ ratio in splenic T-lymphocytes has not been confirmed. Initially it was shown that PEM had no effect on the ratio of CD4+/CD8+ T-cells in the spleen (Woodward & Miller, 1991), however, two more recent studies contradict these initial findings. Taylor and colleagues (1997) have shown that the percentage of CD4+ cells is increased in adult mice fed a 0.5% protein diet for 4 or 6 weeks. Since the percentage of CD8+ remains unchanged, the alteration in CD4+ percentages results in an increase in the ratio of CD4+/CD8+ in the protein deficient

mice. Lee and Woodward (1996) also showed that the CD4+/CD8+ ratio of weanling mice fed a 0.6% protein diet increased compared to controls, due to a significant increase in the percentage of CD4+ cells and little change in the percentage of CD8+ cells. In humans suffering from PEM, populations of CD3+, CD4+ and CD8+ cells were significantly decreased from the controls, although the ratio of CD4+/CD8+ was not significantly different (Abbott, et al, 1986). More research is needed regarding the effect of protein deficiency on T-lymphocyte subpopulations.

Glutathione

Another nutritional deficiency that occurs in conjunction with PEM, and that may contribute to impaired immune defense, is a deficiency of sulfur amino acids, such as cysteine (Bray & Taylor, 1994; Taylor et al, 1997). Sulfur amino acids are generally the most rate limiting amino acid in the diet, and thus protein deficiency often occurs as a result of sulfur amino acid deficient diets. Dietary sulfur amino acid deficiency (also referred to as thiol deficiency) is often used as a model for PEM (Taylor et al, 1997). One of the sulfur amino acids, cysteine is an important structural component of a tripeptide called glutathione (γ-glutamyl-cysteinyl-glycine), which accounts for 90% of the intracellular non-protein thiols (Baker, 1992). The availability of sulfur amino acids such as cysteine, is a major determinant for maintaining hepatic glutathione concentrations within a normal range, and is largely determined by the protein content of the diet (Bray & Taylor, 1994). Cysteine can also be synthesized in the body by the conversion of the sulfur-containing essential amino acid methionine. Sulfur amino acid deficient diets decrease the amount of cysteine that is available for glutathione synthesis

and therefore can be used to study the effects of decreased cellular glutathione on various parameters.

Research regarding the effect of glutathione deficiency on the immune system has recently received much attention due to the discovery that HIV-seropositive individuals have decreased levels of cysteine and glutathione in their plasma and T-lymphocytes (Eck et al, 1989; Staal et al, 1992). It has been suggested that this may increase the risk of opportunistic infection by depressing immune function and accelerate disease progression by potentiating HIV replication (Staal et al, 1992), thus further research is warranted in this area.

Glutathione and Protein-energy Malnutrition

Decreased tissue glutathione has been reported to occur secondary to many diseases associated with PEM, such as AIDS, cancer and alcoholism (Bray & Taylor, 1994). The decreased glutathione status may be a contributing factor in decreasing immune response in malnourished individuals. Mayatepek and colleagues (1993) showed that mean erythrocyte total glutathione concentrations were significantly decreased in children with PEM, compared to controls.

Functions of Glutathione

Glutathione serves many roles in the body and is crucial for cell survival and function. It acts as a reducing agent and an antioxidant, serves as a reservoir for cysteine and may be involved in cell cycle regulation (Deneke & Fanburg, 1989). Glutathione is

also a cofactor for several enzymes and is required for the synthesis of DNA precursors (Staal et al, 1992).

One of the important metabolic roles of glutathione is participation in oxidation-reduction reactions (Figure 1). Under normal conditions, the majority of cellular glutathione is in its reduced state (GSH). GSH functions in reducing intracellular oxidants (free radicals, metabolically active intermediates), thereby protecting the cell from oxidative damage that would otherwise be caused by these agents (Staal et al, 1992). GSH also has been shown to be essential in protecting DNA and other nuclear structures from chemical injury (Bellomo et al, 1992). GSH also modulates enzyme activity, (including enzymes involved in the signal transduction pathway of T-lymphocytes), and various cellular functions (ie. transcription factor binding) by contributing to intracellular redox status (Droge et al, 1994).

GSH also serves as a reservoir for cysteine in cells. Due to the fact that cysteine itself is toxic to cells, it must be stored in a non-toxic form within cells and released as it is needed during the post-absorptive phase. In fact, liver GSH in mice has been shown to increase after a meal and slowly decrease as absorption occurs (Jaeschke & Wendel, 1985). Most cell types do not have the capacity to transport GSH directly into cells, however, the substrates for GSH synthesis can be transported into the cell where GSH synthesis can occur. Under most culture conditions, GSH synthesis by cells is limited by the availability of intracellular cysteine. Cysteine, although initially present in the media, rapidly becomes unavailable because it is oxidized to cystine within a few hours unless a reducing agent is present.

Role of 2-Mercaptoethanol in Culture

2-mercaptoethanol (2-ME) is routinely added to culture media to enhance the survival and growth of murine lymphocytes. Numerous studies have shown that 2-ME is involved in the transport of cysteine and cystine across the membrane of lymphocytes (Ishii et al, 1981). Studies have been done with a cell line of mouse lymphoma (L1210) which is known to be thiol-dependent in vitro due to an incapacity to synthesize cysteine. In culture media, cysteine is readily oxidized to cystine, which L1210 cells cannot transport across their membrane. As a result, cellular cysteine and glutathione contents decrease considerably during culture. However, in the presence of 2-ME, the cells are able to utilize the cystine in the medium, resulting in the maintenance of cellular cysteine and glutathione levels during culture. The proposed mechanism of action is that the reaction of the 2-ME with the cystine present in the medium results in the formation of a mixed disulfide of 2-ME and cysteine. The mixed disulfide is then taken up by cells at a high rate. The 2-ME is repeatedly taken up by cells in the form of the mixed disulfide and returns to the medium in its reduced form. This cyclic action of 2-ME allows cells to continually utilize cysteine and also explains why 2-ME is effective even at very low concentrations (10-100 μM) (Ishii et al, 1981).

Not only does 2-ME act to increase the cysteine concentration of cells, it also increases the intracellular glutathione levels (Ishii et al, 1987; Zmuda and Friedenson, 1983), which is probably a result of the fact that glutathione is a reservoir for cysteine. As cysteine transport into cells is increased, the synthesis of glutathione is also increased.

This function of 2-ME may be important for the proliferation of lymphocytes in culture, as glutathione is important for cell proliferation.

Glutathione and Immune Function

Like zinc deficiency, dietary thiol deficiency has also been shown to affect immune function. A recent study showed that splenocytes from mice fed a low protein diet (0.5%) were greatly decreased in number and had decreased intracellular glutathione concentrations compared to splenocytes from mice fed a protein adequate diet (15%) (Taylor et al, 1997). T-lymphocyte proliferation in the low protein group was lower than the control group when splenocytes were cultured in media without thiol supplementation, but increased when cultured in thiol supplemented media. This suggests that glutathione status in vivo and thiol supplementation in vitro modulate the signal transduction pathway for T-lymphocyte proliferation in PEM mice.

Glutathione and Zinc

Research suggests that there may be a relationship between zinc deficiency and glutathione deficiency. Zinc deficient rats have been shown to have significantly lower blood glutathione levels than control rats after ten days on a zinc deficient diet (Mills et al, 1981). An in vitro study using Chinese hamster cells showed that zinc is involved in glutathione metabolism, as zinc increased the activity of glutathione transferase, an enzyme involved in the synthesis of GSH (Seagrave et al, 1983). More recently it has been shown that metallothionein, a zinc-containing protein, is redox regulated and that a natural oxidative chemical process mobilizes zinc from metallothionein, implicating a

role for glutathione in zinc metabolism (Maret, 1994). In metallothionein, zinc is bonded to the sulfhydryl groups of a number of cysteines. It has been postulated that the release of zinc is initiated by the S-thiolation of the cysteine-metal ligands in metallothionein, caused by a reaction with the disulfide form of glutathione (GSSG) (Maret, 1994). These studies suggest that there is a relationship between zinc and glutathione which may affect the T-lymphocyte signal transduction pathway, and ultimately the cell cycle progression of T-lymphocytes.

Zinc Finger Proteins

Cysteine is an important structural component of a class of proteins called zinc fingers, which "have in common the property of binding zinc ions in order to stabilize the structure of a small autonomously folded protein domain" (Klug & Schwabe, 1995). Zinc finger proteins are composed of a zinc ion in association with four cysteine amino acids, or any combination of cysteine and histidine amino acids, forming a finger-like module (Figure 2). Zinc fingers function in binding transcription factors to DNA and regulate the expression of many genes (Evans & Hollenberg, 1988). The role of zinc in these proteins is purely structural, but is crucial for the DNA binding properties of transcription factors. The first transcription factor to be identified as a zinc finger protein was TFIIIA from *Xenopus laevis* oocytes, which is responsible for activating transcription by RNA polymerase (Vallee & Falchuk, 1993). Conclusive evidence supports the requirement of zinc for DNA binding of TFIIIA. Removal of zinc from the zinc finger protein by chelation agents disrupts DNA binding. It is not known how dietary zinc deficiency affects the function of zinc finger proteins.

Due to the close interrelationship between cysteine and zinc in the structure of zinc finger proteins, it may be postulated that dietary zinc deficiency, which results in decreased available zinc, and PEM, which results in decreased available cysteine, may adversely affect the structure of zinc finger proteins. This would result in disruption of DNA binding of transcription factors, ultimately interfering with transcription.

Cell Cycle Progression

The process of cell division involves one cell dividing into two daughter cells by the serial progression through precisely controlled phases of the cell cycle (Figure 3). These phases are called the resting phase (G_0), the first gap phase (G_1), the DNA synthesis phase (S), the second gap phase (G_2) and mitosis (M). Cells in G_0 phase are quiescent, non-proliferating cells. The cells require activation and certain growth factors to enter the G_1, or first gap, phase of the cell cycle, during which rapid protein synthesis takes place. The transition from G_1 phase to the S phase, or DNA synthesis phase, is critical, as it represents a commitment by the cell to cell division. Progression factors such as IL-2 and insulin-like growth factor-1, are required for the cell to enter the S phase. Beyond this critical point, no further external factors are required, so the cell should progress through the rest of the cell cycle unimpeded. This critical S phase is followed by a second gap, or G_2, phase. The final phase is called the M phase because this is when mitosis occurs. (Reddy, 1994).

Zinc and Cell Cycle

Zinc is known to be an essential trace element necessary for cell proliferation, as it is a cofactor for various enzymes involved in DNA transcription and translation, such as thymidine kinase (Prasad, 1996). Both dietary zinc deficiency in animals and decreased zinc availability in cell cultures resulted in decreased thymidine kinase activity. It seems likely that regulation of the expression of this enzyme is closely linked to the requirement of zinc during growth of animal cells (Chesters et al, 1993).

Zinc is essential for DNA synthesis and cell proliferation. Decreased production of interleukin-2 (IL-2) or decreased responsiveness to IL-2 may also be a contributing factor to decreased cell proliferation and immune function in zinc deficiency, as zinc deficient animals have been shown to have impaired IL-2 production (Mengheri et al, 1995; Moulder and Steward, 1989; Dowd et al, 1986) .

Cell culture studies using ^{3}H-thymidine incorporation strongly suggest that zinc is required for cell cycle progression of lymphocytes based on the percentage of proliferating cells. $ZnCl_2$ has been shown to have an in vitro mitogenic effect in cultures of splenic lymphocytes from C57BL/6 mice in the presence of 2-ME and ConA (Pocino et al, 1992).

A study by Chesters and colleagues (1993) using a cell line showed that growing cells have a significant reduction in ^{3}H-thymidine incorporation in the cultures not supplemented with zinc, which was reversed by zinc supplementation. Lack of zinc not only decreased ^{3}H-thymidine incorporation, but also decreased thymidine kinase mRNA. Results of this experiment suggest that there are at least two zinc-dependent steps prior to

DNA synthesis. It was also suggested that the zinc finger proteins involved in transcription of the mRNA may have been adversely affected by the zinc deficiency.

A limited number of studies have addressed the problem of decreased proliferation more specifically, by looking at the effect of zinc deficiency on the various phases of the cell cycle using the flow cytometric technique. Some experiments did show indirectly that zinc is required in the DNA synthesis phase, or S phase, of the cell cycle, as addition of a chelator to cells in culture resulted in a reduction in DNA synthesis which can only be reversed by addition of the zinc to the culture (Chesters et al, 1989). Chesters and coworkers (1989) used a cell line (3T3 cells) of which the cell cycle has been extensively studied and the timing of many crucial events preceding the entry into S phase have been determined. They found that addition of a zinc chelator inhibited thymidine incorporation by up to 90% and that only the addition of zinc was effective in reversing this effect. The major requirement for zinc was timed to be after stimulation of quiescent cells and before the start of S phase. There was also an indication that zinc is required for the transition from G_1 to S phase. Another study by Chesters and Boyne (1991) also suggested that zinc is required for the progression of untransformed cells into S phase. As a result, the onset of S phase will be delayed.

There is very limited data regarding the effects of dietary zinc deficiency and cell cycle progression, particularly in regard to the specific phases of the cell cycle. One study was found in which the effect of zinc deficiency on ConcanavalinA (ConA) induced rat spleen lymphocyte proliferation was evaluated (Kramer, 1984). This study found that zinc deficiency only caused minor changes in mitogen-induced proliferation.

Numerous other studies were found which investigated the effects of dietary zinc deficiency on the production of IL-2 in culture. Decreased IL-2 production may be an indication of delayed onset of S phase, as IL-2 is a progression factor. The majority of studies regarding the effect of zinc deficiency on IL-2 production and function support the argument that dietary zinc deficiency causes a decreased IL-2 production (Mengheri et al, 1995; Tanaka et al, 1990; Moulder & Steward, 1989; Dowd et al, 1986).

More research is needed regarding the effect of dietary zinc deficiency on T-lymphocyte proliferation, and more specifically, on the effect of dietary zinc deficiency on the various stages of the cell cycle.

Glutathione and Cell Cycle

There is considerable evidence that glutathione is required for cells to progress through the cell cycle. Most research supports the view that glutathione is required for an early event in cell activation (Kavanagh et al, 1990) and that it is required for cells to enter S phase (Messina, 1989; Iwata et al, 1994; Poot et al, 1995). Kavanagh and coworkers (1990) sorted cells on the basis of glutathione content and showed that cells with high glutathione content had a higher percentage of cells capable of entering the cell cycle than cells with a low glutathione content. A more recent study (Iwata et al, 1994) also showed that cells in a thiol free media arrested in the G_0/G_1 phase of the cell cycle.

Hamilos and colleagues (1991) found that it is in the late activation stage that glutathione is involved, as the early activation events (ie. early G_0 - G_1) such as IL-2 production and IL-2 receptor expression are not affected by glutathione depletion.

Therefore, they suggest that a low cellular glutathione content does not affect the cell cycle through an effect on IL-2, but by some other means.

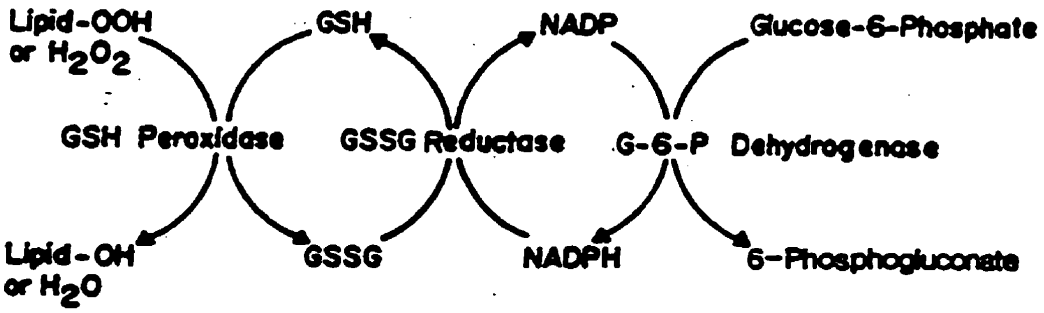


Figure 1. Oxidation and reduction of glutathione.

(Source: Deneke & Fanburg, 1989)

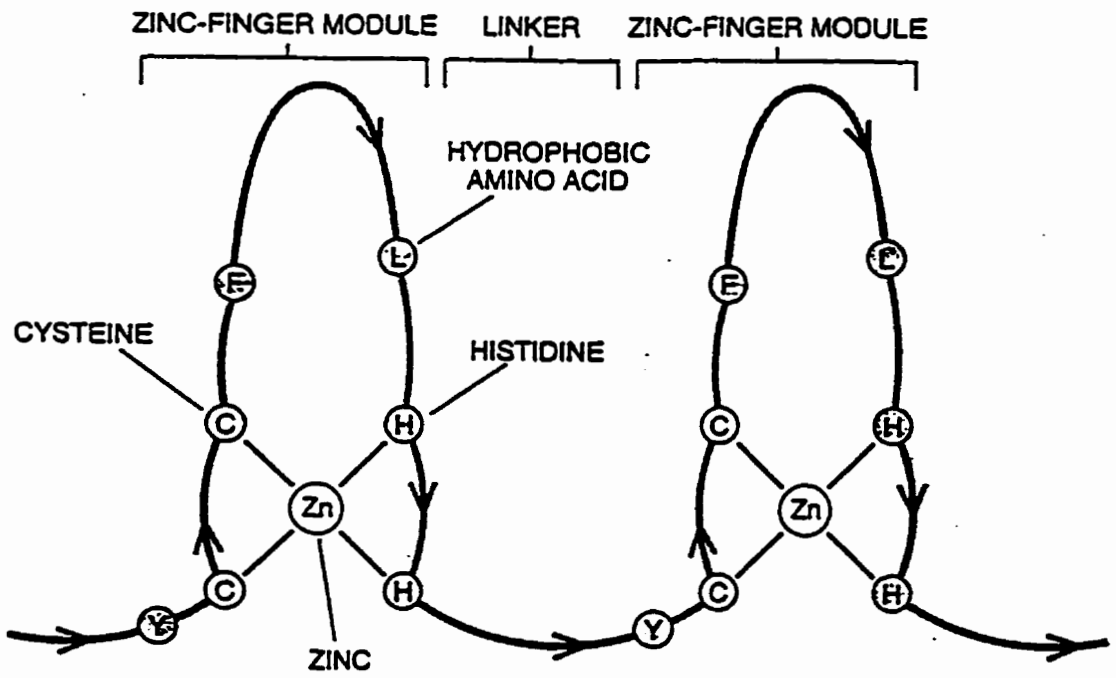


Figure 2. Proposed model of zinc finger proteins.

(Source: Rhodes & Klug, 1993)

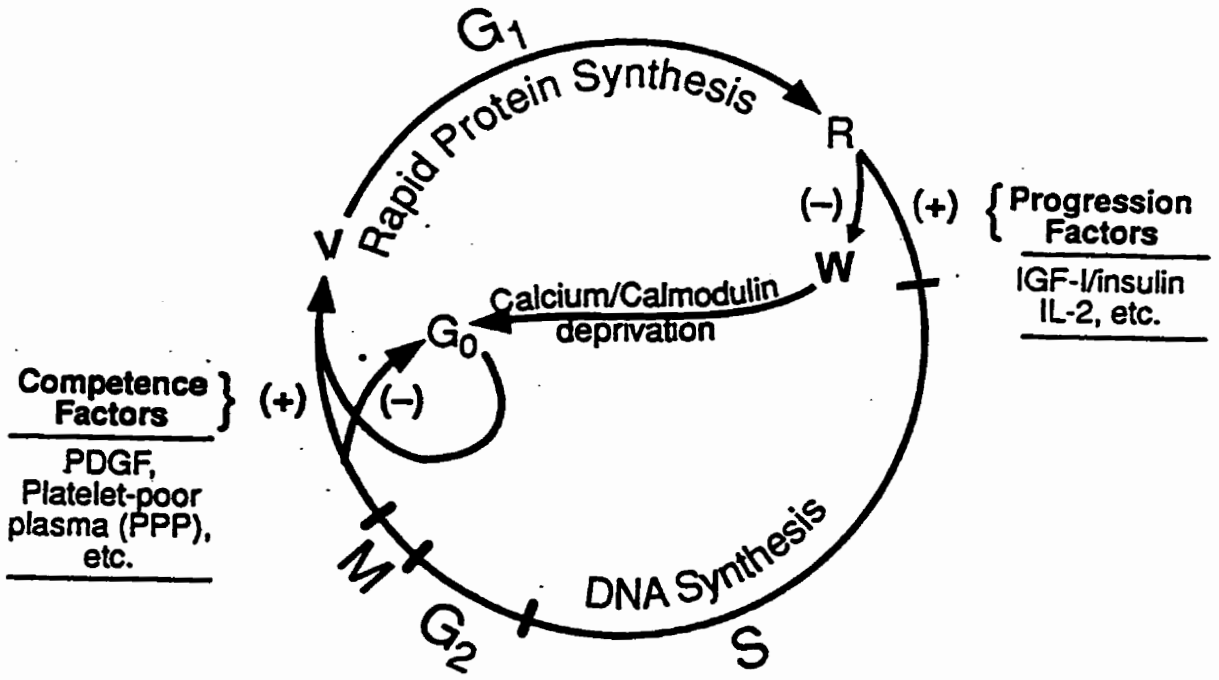


Figure 3. Cell cycle model.

(Source: Reddy, 1994)

II. STUDY RATIONALE

The study of the effects of dietary zinc deficiency has received much attention over the past 30 years, and although a lot of progress has been made in this area, there still are many questions to be answered. Due to the fact that zinc deficiency is often associated with PEM, it is important to study these two deficiencies in conjunction, as well as separate deficiencies. With the use of the advanced technology that is currently available, such as flow cytometry, and the increasing availability of various antibodies, hopefully more questions will be answered regarding the molecular mechanism of zinc function.

Based on experiments using ^{3}H-thymidine, a method commonly used to indicate the percentage of proliferating cells within a culture, zinc has been shown to be required for cell cycle progression. Zinc is required for DNA synthesis, as zinc deficiency has been shown to adversely affect the activities of enzymes involved in DNA synthesis, such as thymidine kinase (Prasad, 1996). However, decreased zinc metalloenzyme activity has generally not explained the signs of zinc deficiency at a molecular level. It is also possible that zinc deficiency may affect cell proliferation through the involvement of zinc in the structure of zinc finger proteins, which are found in the T-lymphocyte signal transduction pathway. Thus it is hypothesized that dietary deficiencies of zinc and/or sulfur-amino acids may adversely affect the progression of T-lymphocytes through the cell cycle via zinc and glutathione status affecting the function of zinc finger proteins.

The overall objective of this thesis was to investigate the effects of dietary zinc deficiency and PEM, alone and in combination, compared to a control group fed a nutritionally adequate diet, on murine splenic T-lymphocyte cell cycle progression and T-

lymphocyte subpopulations, immunological parameters (spleen weight and the number of splenocytes/spleen) and zinc status (serum and femur).

The specific objectives regarding cell cycle progression were to determine which stage of the cell cycle of T-lymphocytes is affected by dietary deficiencies of zinc and protein, alone and in combination, and also to manipulate the thiol status of the culture by the addition or elimination of 2-ME. These objectives address two different components of interest. The one component involves studying the effect of dietary manipulation of zinc and thiol status on the cell cycle, while the other component involves studying the effect of in vitro thiol manipulation of the cell culture conditions, the combination of which may provide interesting information regarding the role of zinc and thiol in the cell cycle. In order to study the cell cycle in this specific nature, a flow cytometry procedure for BrdU incorporation into murine splenic T-lymphocytes in culture had to be optimized, as the procedure for BrdU incorporation varies with different cell types. (See Method Development section in Appendix A for more details.)

Previous studies have addressed the effect of dietary zinc deficiency and its effects on the mitogenic response of T-lymphocytes in culture. However, these studies used a method involving the incorporation of ^{3}H-thymidine into the DNA of proliferating cells and measuring the total ^{3}H-thymidine content of cells to determine the percentage of proliferating cells. This method provides a general indication of proliferation, but gives no information as to which specific stages of the cell cycle are affected by the zinc deficiency. Studies using cell lines have shown that zinc is required in culture for the progression of cells into S phase, or the DNA synthesis phase, of the cell cycle. Flow cytometry analysis of cells that have been cultured in the presence of BrdU and stained

with a flourescent-labeled anti-BrdU antibody and propidium iodide can be used to study cell cycle progression in more detail. This type of analysis has not been used to study spleen cells originating from mice fed diets varying in their nutritional content.

III. MATERIALS AND METHODS

Animals and Diet

Female C57BL/6 mice (Charles River Laboratories, St. Constant, PQ) were obtained at 8 weeks of age. They were housed in pairs and fed nutritionally complete standard mouse chow until they reached immunological maturity at approximately 4 months of age. At that time they were weighed, randomly assigned to one of five treatment groups and transferred to stainless steel hanging cages with wire mesh bottoms. The mesh bottom limited recycling of zinc by allowing feces and urine to drop to the tray of shavings below. Also, to reduce zinc contamination, all mice on Zn deficient diets were housed on the upper rows of the cage rack. The mice were housed individually during the study under conditions controlled for temperature (21-23°C), humidity (55%) and light cycle (14 hours light/10 hours dark). All mice had free access to distilled water which was provided in plastic bottles with stainless steel sipper tubes. The mice were fed their respective dietary treatments for a period of 4 weeks, during which time they were weighed weekly, except for the mice in the energy restricted group, which were weighed daily. Animal care was provided in accordance with the protocol approved by the Local Animal Care Committee (University of Manitoba).

Two feeding trials were conducted due to the fact that each mouse spleen did not provide enough cells for all analysis procedures planned and also because cell preparation had to be performed on the same day as termination, as the cells could not be frozen. Also due to the fact that a limited number of samples could be analyzed on a given day, the mice in each experiment were started on the experimental diets in a

sequential manner. Five mice were started on each day (one mouse from each treatment group), and only two groups were started each week. For Experiment 1, the sample size was 7. For this experiment baseline mice were terminated at the time that the first group of mice were transferred to the treatment diets. Seven mice were randomly selected to be terminated to represent the baseline levels for the parameters measured. For Experiment 2, the sample size was 8 and baseline parameters were not measured.

The acclimatization protocol for the two experiments varied slightly. In the first experiment, the mice assigned to treatment groups were transferred directly from the shoebox cages where they were receiving standard pelleted chow to the hanging cages where they started receiving powdered treatment diets. However, due to the rather large weight loss observed in the first week on the treatment diets, the method was altered for the second experiment in an attempt to acclimatize the mice to the hanging cages prior to introducing treatment diets. Therefore, in Experiment 2, the mice were weighed and randomly assigned to treatment groups at 4 months of age and then transferred to hanging cages in which they were fed powdered control diet for one week. After the one week acclimatization period, mice were weighed and transferred to clean hanging cages and fed their respective treatment diets for 4 weeks.

The dietary treatment groups included: ZnDF&LP (<1 ppm zinc, 2% protein), ZnDF (<1 ppm zinc, 15% protein), LP (30 ppm zinc, 2% protein), and CTRL (30 ppm zinc, 15% protein). One treatment group, referred to as the energy restricted (ER) group, was fed the nutritionally complete control diet (30 ppm Zn, 15% protein) in restricted amounts, in an attempt to match the average weight gain or loss of this group with that of the ZnDF mice. The rationale for including this group is that zinc deficiency over a

prolonged period of time has been shown to cause a decrease in appetite. Therefore, the ER group was meant to serve as a control group for the ZnDF group to interpret whether differences seen are due to zinc deficiency per se or whether they are a result of decreased energy intake.

The mice were fed powdered diets based on the AIN-93M formulation (Reeves, 1993). All groups, aside from the ER group, were allowed to feed ad libitum from glass jars. The ER group was fed a weighed amount of control diet (2.5-4.0 g) in glass jars. Diet ingredients were purchased from Harlan Teklad (Madison, WI), with the exception of the soy oil (Vita Health, Winnipeg, MB). The diet composition is shown in Table 1. The zinc content of the diets was verified by atomic absorption spectrophotometry as described under the zinc analysis section.

Tissue Collection

Mice were terminated by carbon dioxide asphyxiation at the end of the 4 week feeding trial, followed by cervical dislocation according to the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993). The animals were weighed and decapitated for the collection of trunk blood. The blood was stored on ice prior to centrifugation (1290 x g for 15 minutes at 4°C, Beckman Model TJ-6 centrifuge, Mississauga, ON). Spleens were removed by aseptic technique and weighed. Splenocyte preparation was conducted immediately. The hind legs were dissected and frozen at -20°C for femur zinc analysis.

Splenocyte Preparation

All reagents (molecular biology grade) were purchased from Sigma Chemical Company (St. Louis, MO), with the exception of acid solutions, solvents and standard laboratory materials which were purchased from VWR Canlab (Mississauga, ON) or Fisher Scientific (Nepean, ON), unless otherwise specified. Splenocytes (i.e. a suspension of mononuclear cells) were prepared by discontinuous gradient centrifugation using Lympholyte M (Cedarlane Laboratories, Hornby, ON) under aseptic conditions in a Nuaire biological tissue culture hood (Plymouth, MN). Spleens were placed in a round petri dish containing 5 ml of either sterile phosphate buffered saline (PBS) pH 7.4 (80.0 g/l NaCl, 2.0 g/l KCl, 11.5 g/l $Na_2HPO_4 \cdot 7H_20$, 2.0 g/l KH_2PO_4) supplemented with 2% bovine fetal calf serum (FCS, Gibco BRL, Life Technologies, Burlington, ON) for Experiment 1 or sterile RPMI-1640 media (Gibco BRL) for Experiment 2. Spleen cells were removed by gently scraping from the spleen using two bent sterile needles. The spleen sack was discarded and the cells, which included mononuclear and polymorphonuclear cells, erythrocytes and dead cells, were suspended in the RPMI-1640. This cell suspension was gently layered over 5 ml Lympholyte M in a 15 ml plastic conical Falcon tube and centrifuged at 1500 x g for 20 minutes at room temperature (Beckman GS-6 centrifuge). Centrifugation over Lympholyte M reduces polymorphonuclear cells, erythrocytes and dead cells by about 80%, as these cells are pelleted, while the mononuclear cells (which contains the T-lymphocytes of interest) are suspended in an interphase layer. This interphase layer was removed using a glass pipet.

To label cells for flow cytometry in the first experiment, the erythrocyte contamination was further reduced by resuspending the cells from the interphase layer in

2 ml of Tris-buffered ammonium chloride solution (working solution 90 ml 0.16 M NH_4CL, 10 ml 0.17 M Tris pH 7.65; adjusted to pH 7.2) and gently inverting for approximately 2 minutes. This promotes erythrocyte lysis, leaving a more pure mononuclear suspension. The cell suspension was then diluted with 3 ml PBS/2% FCS and centrifuged at 400 x g for 10 minutes. This step was repeated once more to further reduce the erythrocyte population. The cells were washed once at 400 x g for 10 minutes and resuspended in a known volume (2-6 ml) of PBS/2% FCS for cell counting.

For the cell culture work conducted in Experiment 2, the erythrocyte lysis step with Tris-buffered ammonium chloride was omitted. After centrifugation with Lympholyte M, the interphase layer of mononuclear cells was removed and resuspended in 8 ml RPMI-1640. The RPMI-1640 cell suspension was centrifuged at 400 x g for 10 minutes at room temperature. The supernatant was discarded, the cell pellet was resuspended in 8 ml RPMI-1640 and the centrifugation was repeated. The cells were then resuspended in a known volume (1-6 ml) of RPMI-1640 for cell counting.

The total nucleated spleen cell count was determined using an AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY) following the directions provided for the counting of white blood cells. A 10 µl aliquot of the cell suspension was added to 990 µl of 5% acetic acid (dilution factor = 100). The purpose of the acetic acid was to lyse any remaining erythrocytes present in the sample, leaving only mononuclear cells visible, thus simplifying cell counting. A 10 µl aliquot of the acetic acid/cell suspension was loaded onto each counting chamber.

Calculation:

Average cell count x dilution factor (100) x 10 (0.1 mm depth) x 10^3 = total # cells

Cells were also counted using the trypan blue exclusion method to determine cell viability. Trypan blue is a dye which is taken up by nonviable cells and excluded by viable cells. Therefore, cells that appear clear and unstained are counted as viable cells while cells that appear blue are counted as non-viable cells. Splenocyte preparations for both experiments had >95% viable cells, and were adjusted to $1x10^7$ viable cells/ml using RPMI-1640.

Determination of T-Lymphocyte Subpopulations

i) Cell labeling

Two-color flow cytometry was used to determine the percentages of the various subpopulations of T-lymphocytes (ie. $CD4^+$, $CD8^+$ and $CD3^+$) and also to determine the percentage of unstimulated T-cells expressing CD25, or IL-2 receptor. The procedure described below was conducted in dim lighting because of the light-sensitive nature of the flourescent antibodies. Following the splenocyte preparation previously described, 100 µl of the splenocyte/PBS/1% FCS suspension was aliquoted into 1.5 ml microcentrifuge tubes ($1x10^6$ cells/tube) and incubated with 0.5 µg of the appropriate antibody or isotype control for 40-60 minutes at 4°C. Antibodies and controls used were: fluorescein (FITC) anti-mouse CD25 (clone PC61.5.3) with FITC rat IgG1 control (clone LO-DNP-1, Serotec Ltd, Oxford, England); FITC anti-mouse T3 complex CD3ε

(clone 145-2C11) with FITC hamster IgG control; R-phycoerythrin (PE) anti-mouse Ly2 (CD8a, clone YTS 169.4) with PE rat IgG2b control; Tri-Color anti-mouse CD4 (clone CT-CD4) with Tri-Color rat IgG2a control. All antibodies and controls were supplied by Cedarlane Laboratories Ltd, (Hornby, ON) unless otherwise specified. The sample combinations for two-color analysis were: CD8/CD3, CD4/CD3, CD8/CD25 and CD4/CD25 and their respective control samples. Following incubation with the antibodies, 400 µl of cold PBS/0.5% BSA was added to each sample. The samples were then centrifuged at 14 000 RPM (Eppendorf 5414 centrifuge) for approximately 2 minutes. The supernatant fraction was then carefully aspirated and the pellet was resuspended in 400 µl of PBS/0.5% BSA and the centrifugation was repeated. The supernatant fraction was again carefully aspirated and the cells were resuspended in a fixation solution of 1% paraformaldehyde in PBS (pH 7.2). The cells were then stored at 4°C overnight and transported the next morning to the Flow Cytometry Laboratory at the University of Manitoba (Bannatyne Campus) for analysis.

ii) Flow cytometry analysis

Flow cytometry analysis was performed using an EPICS 753 cell sorter (Coulter Electronics Canada, Inc, Burlington, ON) with argon ion laser excitation set at 488 nm (500 mW). The three flourescence emissions were first split with a 590 nm dichroic short pass filter, and the reflected Tri-Color flourescence detected through a 665 nm long pass filter. The remaining transmitted flourescence was further split with a 550 nm dichroic long pass filter with the FITC and PE flourescence signals detected through a 525 nm and 575 nm band pass filters respectively (Figure 4). Control histograms were

derived from cells treated with isotype matched reagents while color compensation adjustments were based on test samples stained with each of the three flourochromes separately. Forward versus side light scatter histograms were collected in order to identify and set up bit map gates for single intact lymphocytes; all flourescence histograms were based on 5000 gated events. Analysis was performed using the Quadstat analysis program included in the instrument operating system. The lymphocyte population was gated on in order to determine the percentage of lymphocytes that were CD3+. The percentage of CD4+ and CD8+ T-cells was determined by gating only on the CD3+ cells.

Cell Cycle Analysis by BrdU Incorporation and Flow Cytometry

Bromodeoxyuridine (BrdU) is a uridine derivative and an analog of thymidine that can be incorporated into DNA in place of thymidine. Anti-BrdU is an antibody that is used to detect the BrdU that has been incorporated into DNA of cells that have undergone DNA synthesis in the presence of BrdU. The proportion of cells in the S-phase of the cell cycle can then be determined by flow cytometry. When BrdU and anti-BrdU are used in conjunction with propidium iodide (PI), which stains for total cellular DNA, the G_0/G_1, S and G_2/M phases of the cell cycle are clearly distinguishable by flow cytomtery.

i) Culturing cells

Complete growth medium was prepared under sterile conditions according to the following recipe: 87.7% RPMI-1640, 10% heat-inactivated FCS, 1%

penicillin/streptomycin, 0.33% 2-mercaptoethanol (0.01516M) in PBS. Growth media deficient in 2-mercaptoethanol contained the same components, except for a slight increase in RPMI-1640 (88%), as it replaced the 2-mercaptoethanol. Medium (1.90 ml) added to each well of a 24-well tissue culture plate (Falcon #3047, Becton Dickinson). An aliquot of 100 µl of cell suspension (approximately $3x10^6$ cells) was added to each well. After all cells were plated, ConA was added to stimulate the T-lymphocytes (other than wells intended to be unstimulated) at a concentration of 5 µg/ml (ie. 10µl/well of a 1µg/µl solution). Plates were then covered and incubated at 37°C and 5% CO_2 (Napco incubator, model 5410, Precision Scientific, Chicago, IL). After 44 hours of culture, 40 µl of 1 mM (BrdU) were added to each well (final concentration of 20 µM), and the incubation continued for another 4 hours.

ii) Harvesting cells

Cells were harvested from culture 4 hours after the addition of BrdU (total of 48 hours of culture). The media was carefully removed from the wells with a pipet and 1 ml of cell dissociation solution (C 1419, Sigma Chemical Co., St. Louis, MO) was added to each well. The plate was allowed to sit at room temperature for 5-10 minutes, during which time each well was gently scraped with a plastic cell scraper to aid in removing adherent cells from the bottom of the plate. Then the contents of the wells were transferred to 15 ml plastic conical Falcon tubes and 1 ml PBS/1% bovine serum albumin (BSA) was added to each harvested sample. For the stimulated samples, four wells were plated per mouse. Upon harvesting the cells, these four wells were combined and two 1 ml aliquots were transferred to separate tubes to serve as duplicate samples.

For the unstimulated wells, one well was plated per mouse, therefore, each well represented one sample.

iii) Procedure for staining with anti-BrdU

The method used for cell cycle analysis was based on the procedure by Becton Dickinson (Source book section 3.80.1). The harvested cells were centrifuged in the cell dissociation solution/PBS/1% BSA solution for 15 minutes (500 x g) at room temperature. The cells were resuspended in 100 µl of normal saline and placed on ice. Prior to starting this procedure, 5 ml of 70% ethanol had been aliquoted into 15 ml plastic conical Falcon tubes and stored at -20°C. At this stage, the cells suspended in normal saline were slowly added, a few drops at a time, to the cold ethanol while maintaining a vortex. The tubes were then incubated on ice for 30 minutes, followed by centrifugation at 500 x g for 10 minutes at 10°C. The supernatant fraction was carefully aspirated, and the pellet was resuspended by adding 500 µl of 2 N HCl with 0.5% Triton X-100, a few drops at a time, while maintaining a vortex. The samples were incubated at room temperature for an additional 30 minutes and subsequently centrifuged at 500 x g for 10 minutes at room temperature. The cells were resuspended in 500 µl of 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, pH 8.5 and centrifuged at 500 x g for 10 minutes. Following this centrifugation, the cells were resuspended in 50 µl of PBS/1% BSA and transferred to 1.5 ml plastic microcentrifuge tubes. All procedures after this point were carried out in dim lighting to preserve the flourescence of the antibody. To each tube, 20 µl of FITC-conjugated Anti-BrdU (Clone B44, Becton Dickinson, San Jose, CA) was added and the samples were incubated at room temperature for 30 minutes. The cells were then

centrifuged at 14 000 RPM (Eppendorf 5414 centrifuge) for approximately 1 minute, and resuspended in 300 µl of PBS containing 5 µg/ml of propidium iodide. The samples were then transferred on ice to the Flow Cytometry Lab at the University of Manitoba (Bannatyne Campus) where they were analyzed within 3 hours.

iv) Flow cytometry analysis

Flow cytometry analysis was performed on an EPICS 753 cell sorter (Coulter Electronics, Inc) with laser excitation set at 488 nm (500 mW). Forward vs. side light scatter histograms were used to gate on intact cells and eliminate debris, while peak vs. integrated propidium iodide derived flourescence signals were used for doublet discrimination gating (Figure 5 a-d). The flourescence signals derived from each cell were split with a 550 nm dichroic long, with the PI and FITC signals detected through 630 nm long pass and 525 nm bandpass filters, respectivley (Figure 6). Bivariate flourescence histograms of 64x64 channel resolution were based on 5000 events satisfying the light scatter and doublet discrimination gating criteria. Cell cycle determinations were performed using the Quadstat analysis program included in the instrument operating system.

Analysis of Zinc Status

Zinc content of femur, serum and treatment diets was assessed by atomic absorption spectrophotometry. A scalpel blade was used to remove all musculature and connective tissues from thawed femurs. A technique referred to as wet-ashing was used

to digest femurs and diets (Clegg et al, 1981). Wet weights of the femurs were taken prior to drying for 48 hours in an 85°C oven. Pyrex tubes were soaked in a 15-20% nitric acid solution for a minimum of 24 hours to remove traces of zinc from the tubes and rinsed with distilled, deionized water. Each femur was placed in a tube containing 1 ml 70% nitric acid and allowed to digest for 24 hours at room temperature, followed by heating for 48 hours at 85°C. The digested femurs were then diluted to 20x with distilled, deionized water and analyzed by atomic absorption spectrophotometry, using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, ON).

Femur zinc concentration calculation:

$$\frac{\text{Sample zinc concentration (}\mu\text{g/ml) x dilution factor (2) x volume (10ml)}}{\text{Femur dry weight (g)}} = \text{Femur zinc } \mu\text{g/g dry weight}$$

Diets were analyzed for zinc content by digesting 1 g of each diet in 2 ml 70% nitric acid at room temperature. Zinc deficient diets were diluted 5x and zinc-adequate diets were diluted 50x with distilled, deionized water prior to analysis.

Serum zinc was assessed directly by a 10 to 20 fold dilution of the sample. Approximately 100 µl of sample was required, and thus some samples were pooled.

Serum zinc concentration calculation:

Sample zinc concentration (µg/ml) x dilution factor = Serum zinc µg/ml

Zinc standards (0.1-1 ppm) were prepared from zinc atomic absorption standard (1000 ppm, # H595-01 Mallinckrodt, Paris, Kentucky). Quality control was monitored by digesting 0.1 g bovine liver standard reference material 1577b (U.S. Department of

Commerce, National Institute of Standards and Technology, Gaithersburg, MD) in 1 ml 70% nitric acid and preparing according to the protocol for femurs.

Statistical Analysis

Analysis for main effects was performed using one-way ANOVA (SAS software release 6.04, SAS Institute, Cary, NC). Significant differences among weekly weights were determined by repeated measures ANOVA. For procedures where one block of samples was analyzed per day, the model statement tested for main effects of diet and day. There was no significant main effect of day for all variables analyzed with the exception of some cell cycle analysis results. Significant main effects of day were found for stimulated samples cultured with 2-ME for G_0 phase, early S phase, late S phase and mitosis. For stimulated samples cultured without 2-ME, significant main effects of day were noted for G_0 phase and late S phase. If no main effect of day was found, then ANOVA was repeated with a model statement including only diet, in order to increase the number of degrees of freedom available for mean square error. For cell cycle data, analysis of main effects also included treatment (ie. +2-ME versus -2-ME) and stimulation (ie. stimulated versus unstimulated). When diet was a significant main effect, significant differences between means were determined using Duncan's multiple range test. Differences were considered significant at $p<0.05$.

Table 1

Diet Formulation (g/kg diet)[1]

Ingredient	ZnDF&LP[2]	ZnDF	LP	CTRL
Dextrose	887.5	762.5	877.5	752.5
Egg white	25.0	150.0	25.0	150.0
Soybean oil	40.0	40.0	40.0	40.0
Mineral mix (zinc free)[3]	35.0	35.0	35.0	35.0
Vitamin mix[4]	10.0	10.0	10.0	10.0
Choline	2.5	2.5	2.5	2.5
Zinc premix[5]	0.0	0.0	10.0	10.0

[1]Diet ingredients were purchased from Harlan Teklad (Madison, WI) with the exception of the soybean oil (Vita Health, Winnipeg, Manitoba) and the dextrose (Moonshiners, Winnipeg, MB).
[2]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.
[3]AIN-93 zinc free mineral mix (Harlan Teklad).
[4]AIN-93 vitamin mix (Harlan Teklad).
[5]Zinc premix (11.55 g zinc carbonate/1000 g dextrose).

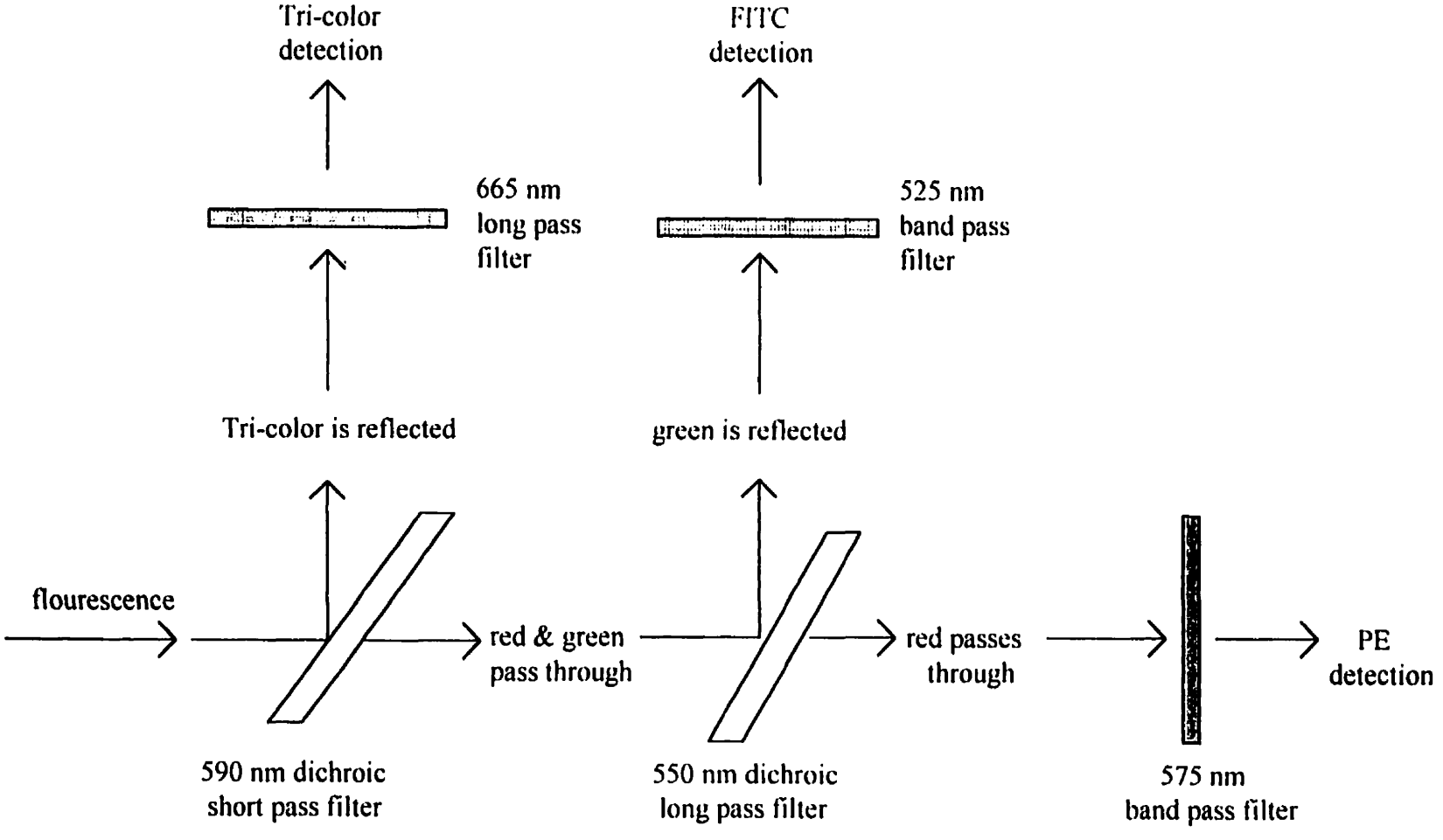


Figure 4. Diagrammatic representation of the use of filters to detect fluorescein isothiocyanate (FITC), Tri-Color and phychoerythrin (PE) by flow cytometry. See text for details.

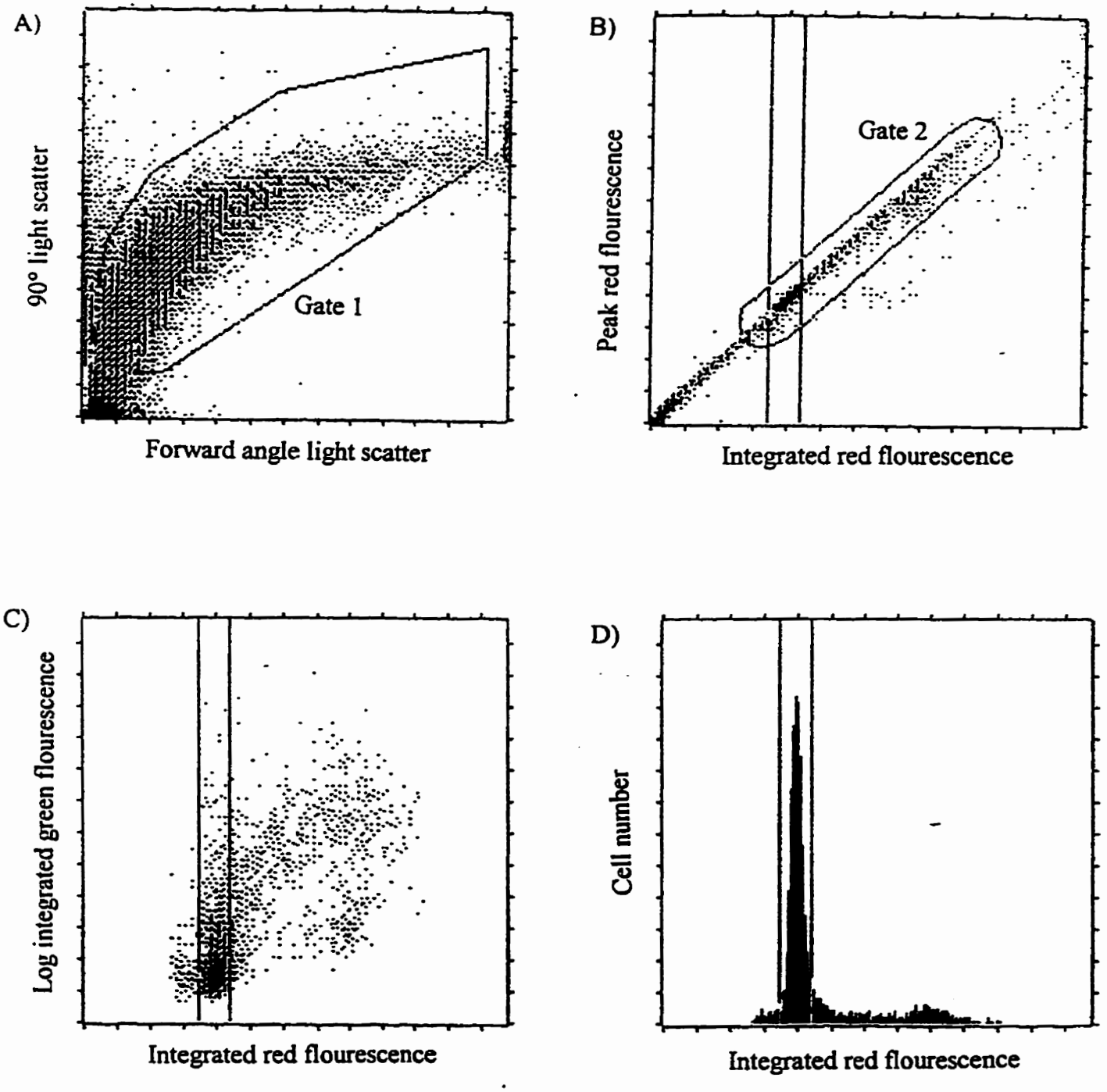

A)
90° light scatter
Gate 1
Forward angle light scatter
B)
Peak red flourescence
Gate 2
Integrated red flourescence
C)
Log integrated green flourescence
Integrated red flourescence
D)
Cell number
Integrated red flourescence

Figure 5. A) Histogram plotting 90° angle light scatter (an indication of cell granularity) versus forward angle light scatter (an indication of cell size). Gate 1 is set to identify intact cells and to eliminate much of the cellular debris. B) Histogram plotting peak red fluorescence versus integrated red fluorescence. This histogram is based only on cells that met the criteria established by Gate 1 of histogram A. The red fluorescence is a result of staining DNA with propidium iodide (PI). Gate 2 is set to include single cells stained with PI and to discriminate against doublets. C) Histogram plotting the log of the integrated green fluorescence versus the integrated red fluorescence of cells stained with PI. This histogram is based on cells that have met the criteria of both Gate 1 and Gate 2. Division of histogram C into quadrants provides information regarding the percentage of cells in each phase of the cell cycle. D) Histogram of cell number versus integrated red flourescence. This histogram is based on cells that have met the criteria of both Gate 1 and Gate 2.

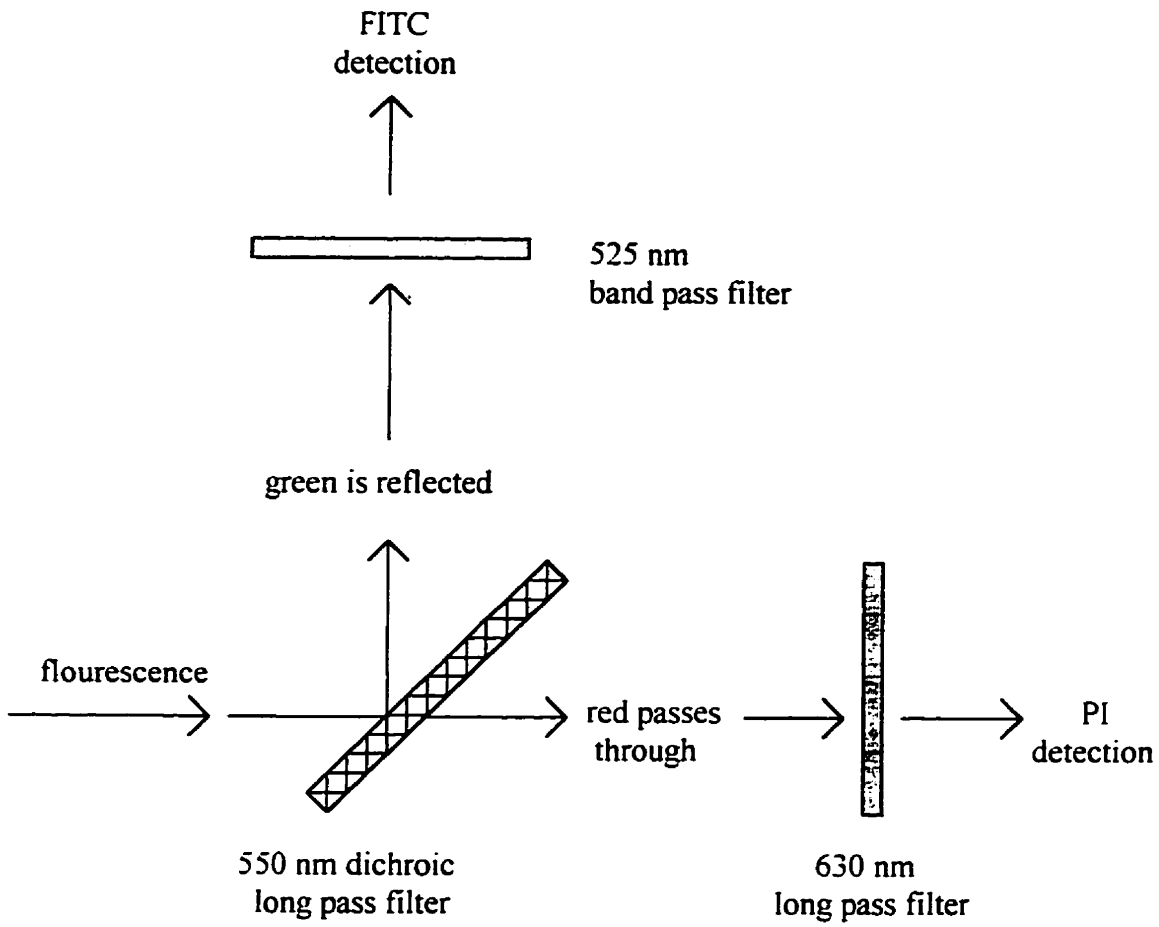


Figure 6. Diagrammatic representation of the use of filters to detect flourescein isothiocyanate (FITC) and propidium iodide (PI) by flow cytometry. See text for details.

IV. RESULTS

Experiment 1

Assessment of Malnutrition and Zinc status

Weight loss is a general indicator of malnutrition and Zn deficiency and was evident in all four deficiency groups when compared to the CTRL group at week 4. Figure 7 graphically displays the weekly weights for mice in Experiment 1. Values for the means ± the standard error of the mean for the weekly weights are presented in Appendix B. All treatment groups experienced weight loss during the first week of the experiment, when moved from group housing (2 mice/cage) in mouse boxes and a laboratory chow diet, to individual housing in stainless steel mesh-bottom cages and a powdered diet. Even though the ZnDF&LP, ZnDF and LP groups weighed less than the CTRL and ER groups after week 1, there were no significant differences in weight until the second week of the feeding trial, at which time the ZnDF&LP and LP groups weighed significantly less than the CTRL group. The ZnDF&LP & LP groups continued to lose weight, and by the third and fourth week of the experiment, their body weight was also significantly different from the ZnDF and ER groups. In addition, two mice receiving the ZnDF&LP diet became so severely malnourished that they had to be terminated prior to the end of the experiment. By week 4, all deficient groups had significantly lower average body weights than the CTRL group.

At baseline, the mean body weight was 23.4 g. At the end of the experiment, the CTRL group had just regained the weight loss experienced during the initial week of the

experiment (2.7 g). See Appendix B. None of the four deficient groups were able to gain the lost weight.

Zinc status was assessed by measuring serum zinc concentrations which are presented in Table 2 and Appendix C. Both the ZnDF&LP and the ZnDF groups had significantly reduced serum zinc concentrations (54.4% and 50.9% lower than the CTRL respectively) compared to the other three groups. The LP and ER groups did not have significantly different serum zinc concentrations than the CTRL group.

Assessment of Immunological Parameters

Two indicators of decreased immunological status are a decrease in spleen weight and a decrease in spleen cell (splenocyte) numbers. Effects of dietary treatment on these parameters are presented in Table 3 and Appendix D. Although there were no significant differences among the deficient groups in regards to spleen weight, all four deficient groups experienced a significant decrease in spleen weight compared to both baseline and CTRL groups. The ZnDF&LP group had the largest decrease (50.7%) in spleen weight compared to the CTRL group. The same trends were observed when spleen weight was expressed as a percentage of total body weight. All four deficient groups had a significantly lower percentage of spleen weight compared to body weight than the baseline and CTRL groups, with the largest decrease (36.7%) observed in the ZnDF&LP group.

Splenocyte counts (splenocytes/spleen x 10^8) of the ZnDF&LP group were significantly lower (54.4%) than the CTRL group. Although the ZnDF, LP and ER groups had lower splenocyte counts than the CTRL group, (lower by 38.1%, 40.5% and

35.8%, respectively), these values were not significantly different from the CTRL group. When spleen cell number was expressed per mg of spleen, no significant differences were observed.

T-Lymphocyte Subpopulations

The effects of dietary treatment on the proportions of CD3+, CD4+, CD8+ and CD25+ T-cells was determined by flow cytometry and are presented in Table 4 and Appendix E. The percentage of T-cells in the splenocyte preparation was determined by the use of the well-known T-cell marker, CD3+. The percentage of CD3+ cells was higher in the deficient groups (15.8%-37.7%) than in either the baseline or the CTRL group.

Anti-CD4 and anti-CD8 antibodies were used to identify the two main subpopulations of T-cells, namely CD4+ T-helper cells and CD8+ T-suppressor/cytotoxic cells. The percentages of CD4+ and CD8+ T-cells were expressed as a percentage of CD3+ splenocytes. There were some differences noted among the groups in regards to the percentage of CD4+ T-cells. The ZnDF&LP group exhibited a significantly lower percentage of T-helper cells when compared to all the other groups. The ZnDF group had a significantly lower percentage of T-helper cells compared to the baseline group, but was not significantly different from the CTRL group. There were no significant differences among the percentages of CD8+ T-cells. The ratio of CD4+/CD8+ did not differ significantly among the groups.

The total percentage of T-cells expressing either CD4+ or CD8+ was significantly higher in the baseline and CTRL groups compared to all the deficient

groups. The ZnDF&LP group had the lowest total percentage of cells expressing either CD4+ or CD8+, and this was significantly lower than both the LP and ER groups.

There were no significant differences among the different dietary groups in regards to the percentage of lymphocytes expressing CD25 (interleukin-2 receptor).

Experiment 2

Assessment of Malnutrition and Zinc Status

The protocol for Experiment 2 varied slightly from that of Experiment 1. In Experiment 2, the mice were acclimatized to the hanging cages and fed powdered CTRL diet for one week before being fed their respective treatment diets. Figure 8 graphically displays the weekly weights for mice in Experiment 2. Values for the means ± the standard error of the means for the weekly weights are presented in Appendix B. At baseline, the mean body weight was 23.2 g. The mean weight after acclimatization was 20.9 g (an average decrease of 2.3 g). All groups experienced a 1.5-2.8 g weight loss during the acclimatization period during which time all mice were fed powdered control diet in hanging cages. By the second week of receiving their respective treatment diets, the ZnDF&LP, LP and ER groups weighed significantly less than the CTRL group. At weeks 3 and 4, the ZnDF&LP and LP groups weighed significantly less than the ZnDF group. Two mice in the LP group were so severely malnourished that they had to be terminated prior to the end of the experiment.

By week four, weight loss was evident in all four deficient groups when compared to the CTRL group. The mean weight of the ZnDF group was significantly less than the mean weight of the CTRL group at week 4. By the end of the four week feeding trial, the

CTRL group was 2.3 g heavier than at baseline (an average of 4.6 g gained after the acclimatization period). None of the other dietary treatment groups returned to their baseline weight. Of the deficient groups, only the ZnDF and ER groups recovered some of the weight lost during the acclimatization period, while the ZnDF&LP and LP groups experienced continual weight loss.

Zinc status was assessed by measuring serum zinc, femur zinc and spleen zinc concentrations. Effects of diet on these parameters are presented in Table 2 and Appendix C. Serum zinc represents short term zinc body pools, while femur zinc is more representative of long term body pools. Both the ZnDF&LP and ZnDF groups were found to have serum zinc concentrations significantly lower than the CTRL and the ER groups (44.9% and 38.5% lower respectively). The LP group also had significantly lower serum zinc concentrations than the CTRL group (33.8% lower) but they were not significantly different from the ER group.

No significant differences were observed in femur zinc or spleen zinc concentrations among the treatment groups.

Assessment of Immunological Parameters

Effects of dietary treatment on spleen weight and splenocyte counts for Experiment 2 are presented in Table 5 and Appendix F. All four deficient groups had a significantly lower spleen weight after four weeks on their respective treatment diets when compared to the CTRL group; however, there were no significant differences among the deficient groups. The lowest spleen weight was observed in the LP group (37.2% lower than CTRL). When spleen weight was expressed as a percentage of total

body weight, the ER group was significantly different from the CTRL group, but not significantly different from the other three deficient groups.

The only group that had spleen cell counts significantly lower than the CTRL group was the LP group (50.7% less than CTRL). The other deficient groups had lower splenocyte counts than the CTRL group (41.6%, 40.7% and 39.7% lower for the ZnDF&LP, ER and ZnDF groups, respectively), however, these differences were not found to be statistically different. When splenocyte count was expressed per mg of spleen, no significant differences were seen among the groups.

Cell Cycle Analysis

The effects of dietary treatment and 2-ME treatment in vitro on the percentage of unstimulated and ConA-stimulated murine spleen cells in each phase of the cell cycle are presented in Table 6 and Table 7, respectively. Stimulation (± ConA) and culture condition (± 2-ME) were found to be significant main effects ($p<0.05$) for all phases of the cell cycle, except for mitosis, where no significant effect of culture condition was present. Figure 9 compares representative cell cycle histograms derived from unstimulated and ConA-stimulated spleen cell cultures. There was no significant effect of diet or culture condition (±2-ME) among unstimulated cultures. Since stimulated cultures were found to be significantly different from unstimulated cultures for all phases, all references to cell cycle analysis results after this point will be for stimulated samples only.

Effects of dietary treatment and 2-ME treatment in vitro on the percentage of ConA-stimulated spleen cells in each phase of the cell cycle are presented in Figure 10.

The mean values ± the standard error the mean are presented in Appendix G. For samples cultured in the absence of 2-ME, there were no significant main effects of diet, however, significant main effects of day were observed for G_0 and late S phases. When means testing was performed, differences among diet groups were observed only for the mitosis phase for samples cultured in the absence of 2-ME (Figure 10e). The LP group had significantly higher percentage of cells in M compared to all the other groups except for the ER group.

Differences in the percentage of cells in each phase of the cell cycle among dietary treatment groups was most obvious in the samples that had been incubated in the presence of 2-ME. Significant day effects were found for a number of the variables, including G_0, early S, late S and total S. Accounting for the effect of day in the model statement, significant main effects of diet were found for the G_0 phase, early S phase, late S phase and total S phase for cells cultured with 2-ME ($p<0.05$).

S phase is also referred to as the DNA synthesis phase because this is the portion of the cell cycle during which DNA synthesis occurs. Analysis of the cell cycle by flow cytometry and BrdU incorporation allows the S phase to be further divided up into early S phase and late S phase. Con-A stimulated spleen cells from the CTRL group, cultured in media with 2-ME, had a significantly higher percentage of cells in S phase (including both early and late S phase) than cells from the other groups (Figure 10a-c). The ZnDF and LP groups had the lowest percentage of cells in S phase when compared to the CTRL group (38.5% and 38.7% lower respectively). When the percentage of cells in S phase is broken down into both early and late S phase, similar results are seen (Figure 10b and 10c). ZnDF&LP, ZnDF and LP groups had significantly fewer cells in both early and late

S phase than the CTRL group. However, there was no difference between the CTRL group and the ER group.

The G_0 phase is also referred to as the resting phase, because cells in this stage of the cell cycle are quiescent, non-proliferating cells. The ZnDF&LP, ZnDF and LP groups had significantly more cells in the resting state when compared to the CTRL group for cells cultured with 2-ME (Figure 10d). Following the same pattern as for the early and late S phases, the ER group was not found to be significantly different from the CTRL group regarding the percentage of cells in the resting phase.

No significant differences were found among the dietary groups for the percentage of cells in mitosis when the cells were cultured with 2-ME (Figure 10e).

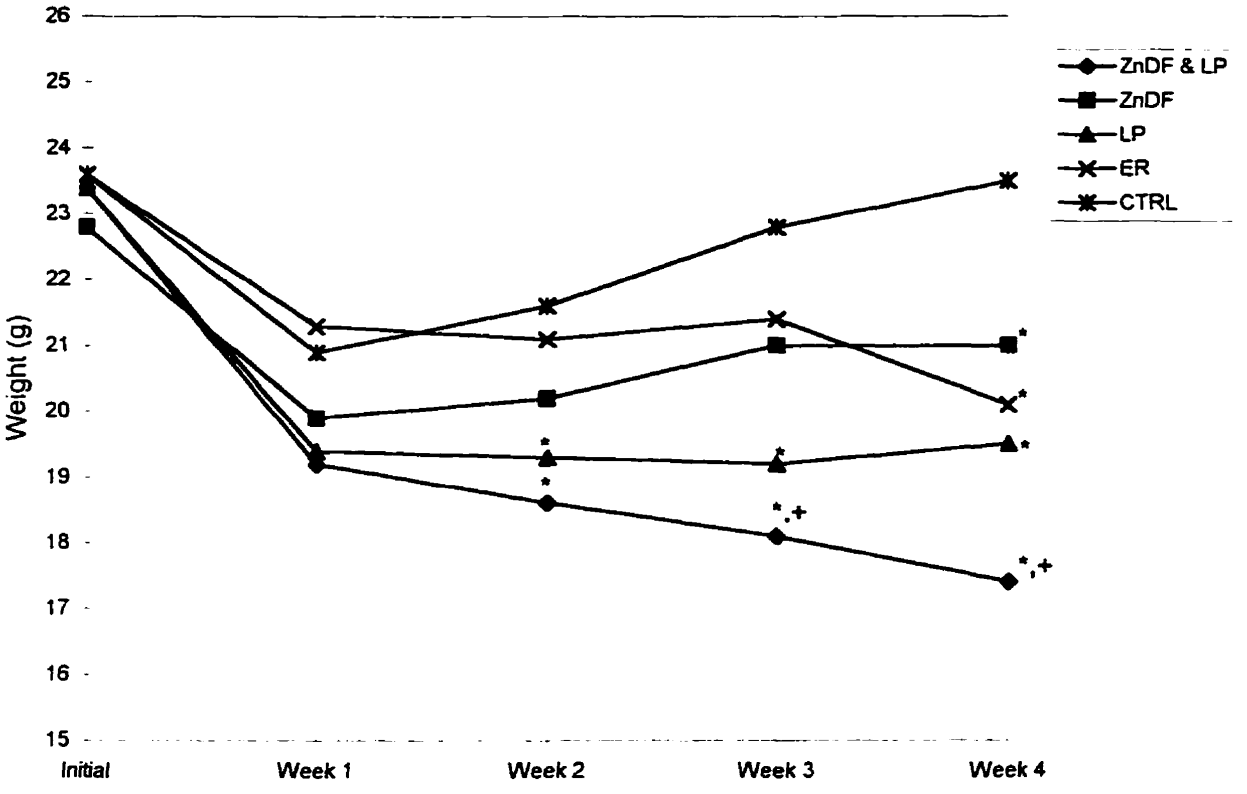


Figure 7. Effects of dietary treatments on weekly weights of mice in Experiment 1. Points represent mean values for n=7, except for ZnDF & LP, where n=5. The pooled standard error for the data set is 1.43. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. * Significantly different than CTRL for a given week. + Significantly different than ZnDF and ER for a given week. ZnDF&LP=Zn deficient and 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

Table 2

Effects of dietary treatments on femur, serum and spleen zinc concentration in mice after 4 weeks[1]

	Treatment Groups[2]					
	ZnDF&LP	ZnDF	LP	ER	CTRL	Pooled SE[3]
Serum Zn (Exp 1)[4] (*ug*/ml)	0.52^{B} ↓54.3%	0.56^{B} ↓50.9%	1.01^{A} ↓11.4%	1.15^{A} ↑0.9%	1.14^{A}	0.11
Serum Zn (Exp 2)[5] (*ug*/ml)	0.75^{C} ↓44.8%	0.75^{C} ↓44.8%	$0.90^{B,C}$ ↓33.8%	$1.22^{A,B}$ ↓10.2%	1.36^{A}	0.74
Femur Zn (Exp 2)[6] (*ug*/g dry wt)	264.1 ↓13.7%	254.8 ↓16.8%	268.2 ↓12.4%	249.4 ↓18.5%	306.2	59.8
Spleen Zn[7] (*ug*/g dry wt)	415.5 ↑13.5%	761.1 ↑107.9%	502.8 ↑37.3%	431.4 ↑17.8%	366.1	349.7

[1]Values are means and percent increase or decrease from CTRL. Main effects of diet were significant for serum Zn in Experiment 1 and 2. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.
[2]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.
[3]To determine the pooled standard error of the mean for a particular diet mean, divide the pooled standard error (SE) for a variable by the square root of n for that diet.
[4]Values are from mice in Experiment 1. n=3, except for ZnDF&LP and CTRL, where n=2 and 4, respectively. Values represent pooled samples.
[5]Values are from mice in Experiment 2. n=7, except for ZnDF&LP, ZnDF and LP, where n=6, 8 and 5, respectively.
[6]Values are from mice in Experiment 2. n=7, except for LP and ER, where n=5 and 8, respectively.
[7]Values are from a separate group of mice fed according to the same protocol as for Experiment 1. n=5 for all groups.

Table 3

Effects of dietary treatments on spleen weight and spleen cell numbers in mice from Experiment 1 after 4 weeks[1]

	Treatment group[2]						
	B	ZnDF&LP	ZnDF	LP	ER	CTRL	Pooled SE[3]
Spleen weight (mg)	104.0[A] ↓9.2%	56.5[B] ↓50.7%	74.9[B] ↓34.6%	70.7[B] ↓38.3%	73.3[B] ↓36.0%	114.5[A]	1.90
% Spleen/Body weight	0.45[A] ↓8.2%	0.31[B] ↓36.7%	0.35[B] ↓28.6%	0.36[B] ↓26.5%	0.36[B] ↓26.5%	0.49[A]	0.07
Splenocytes/ spleen ($x10^6$)	1.56[A,B] ↓27.4%	0.98[B] ↓54.4%	1.33[A,B] ↓38.1%	1.28[A,B] ↓40.5%	1.38[A,B] ↓35.8%	2.15[A]	0.79
Splenocytes/ mg spleen ($x10^6$)	1.50 ↓18.0%	1.60 ↓12.6%	1.79 ↓2.2%	1.71 ↓6.6%	1.59 ↓13.1%	1.83	0.70

[1]Values are means and percent decrease from CTRL. n=7, except for ZnDF&LP, where n=5. Main effects of diet were significant for spleen weight and spleen weight as a percent of body weight. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]B=baseline, ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[3]To determine the standard error of the mean for a particular diet mean, divide the pooled standard error (SE) for a variable by the square root of n for that diet.

Table 4

Effects of dietary treatments on T-lymphocyte subpopulations[1]

	Treatment Groups[2]						
	B	ZnDF&LP	ZnDF	LP	ER	CTRL	Pooled SE[3]
% CD3+[4]	13.8[B] ↓5.5%	18.1[A,B] ↑24.0%	16.9[A,B] ↑15.8%	19.6[A] ↑34.2%	20.1[A] ↑37.7%	14.6[B]	3.8
% CD4+[5]	57.2[A] ↑2.3%	42.9[C] ↓23.3%	49.8[B] ↓10.9%	51.7[A,B] ↓7.5%	51.1[A,B] ↓8.6%	55.9[A,B]	5.5
% CD8+[5]	15.5 ↓7.7%	16.3 ↓3.0%	14.6 ↓13.1%	14.7 ↓12.5%	14.7 ↓12.5%	16.8	3.1
Total % CD4+ and CD8+[5]	72.7[A] 0%	59.2[C] ↓18.6%	64.4[B,C] ↓11.4%	66.4[B] ↓8.7%	65.8[B] ↓9.5%	72.7[A]	4.8
CD4+/CD8+ ratio	3.8 ↑8.6%	2.8 ↓20.0%	3.7 ↑5.7%	3.7 ↑5.7%	3.6 ↑2.9%	3.5	1.0
%CD25+[6]	1.45 ↓1.4%	1.35 ↓8.2%	1.40 ↓4.8%	1.47 0%	1.69 ↑15.0%	1.47	0.4

[1]Values are means and percent increase or decrease from CTRL.. n=7, except for ZnDF&LP, where n=5. There was a significant main effect of diet for CD4+, and total CD4+ and CD8+, and a significant main effect of day for CD3+, CD4+, CD8+ and total CD4+ and CD8+. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.
[2]B=baseline, ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.
[3]To determine the standard error of the mean for a particular diet mean, divide the pooled standard error (SE) for a variable by the square root of n for that diet.
[4]Expressed as % of total splenocytes.
[5]Expressed as % of CD3+ splenocytes..
[6]Expressed as % of total splenocytes.

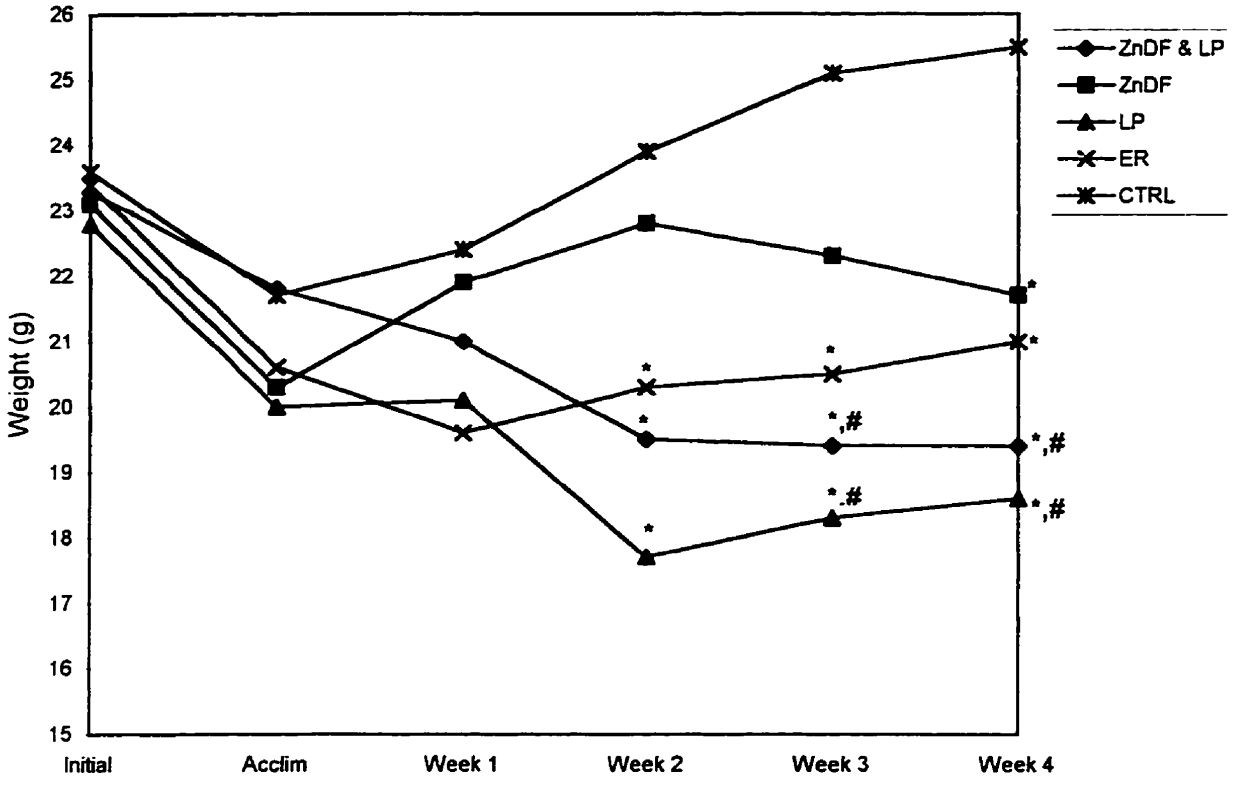


Figure 8. Effects of dietary treatments on weekly weights of mice in Experiment 2. Points represent mean values for n=8, except for LP, where n=6. The pooled standard error for the data set is 1.41. Significant main effects as determined by repeated measures ANOVA were week and diet x week. * Significantly different than CTRL for a given week. # Significantly different than ZnDF for a given week. ZnDF&LP=Zn deficient and 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control. Acclim=weight after acclimatization in hanging cages fed powdered control diet.

Table 5

Effects of dietary treatments on spleen weight and spleen cell numbers in mice from Experiment 2 after 4 weeks[1]

	Treatment group[2]					
	ZnDF&LP	ZnDF	LP	ER	CTRL	Pooled SE[3]
Spleen weight[4] (mg)	78.1^{B} ↓30.3%	87.8^{B} ↓21.6%	70.3^{B} ↓37.2%	73.6^{B} ↓34.3%	112.0^{A}	18.3
% Spleen/Body weight[4]	$0.40^{A,B}$ ↓9.1%	$0.40^{A,B}$ ↓9.1%	$0.37^{A,B}$ ↓15.9%	0.35^{B} ↓20.5%	0.44^{A}	0.07
Splenocytes/ spleen[5] ($x10^{8}$)	$1.22^{A,B}$ ↓41.6%	$1.26^{A,B}$ ↓39.7%	1.03^{B} ↓50.7%	$1.24^{A,B}$ ↓40.7%	2.09^{A}	0.77
Splenocytes/ mg spleen[5] ($x10^{6}$)	1.63 ↓12.8%	1.35 ↓27.8%	1.35 ↓27.8%	1.52 ↓18.7%	1.87	0.90

[1]Values are means and percent decrease from CTRL. There was a significant main effect of diet for spleen weight. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.
[2]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.
[3]To determine the standard error of the mean for a particular diet mean, divide the pooled standard error (SE) for a variable by the square root of n for that diet.
[4]n=8, except for LP, where n=6.
[5]n=7, except for LP, ER, and CTRL, where n=4, 5, and 8, respectively.

Table 6

Effects of dietary treatment and 2-mercaptoethanol treatment in vitro on the percentage of unstimulated murine spleen cells in each phase of the cell cycle[1,2]

Treatment Group[3]	Culture Conditions									
	+2-ME					-2-ME				
	G_0	Early S	Late S	Total S	Mitosis	G_0	Early S	Late S	Total S	Mitosis
ZnDF&LP[4]	88.32 ±2.04	2.55 ±0.66	1.28 ±0.37	3.83 ±1.00	7.85 ±1.29	89.89 ±2.43	2.31 ±0.65	1.21 ±0.55	3.52 ±1.18	6.59 ±1.49
ZnDF	91.89 ±2.04	1.67 ±0.48	0.72 ±0.33	2.39 ±0.79	5.72 ±1.35	91.01	2.39	1.08	3.47	5.53
LP	90.90 ±0.55	2.49 ±0.55	1.00 ±0.11	3.49 ±0.57	5.62 ±1.07	90.63 ±1.18	3.32 ±1.25	1.11 ±0.38	4.43 ±1.59	4.94 ±1.30
ER	91.24 ±2.92	2.97 ±1.62	1.22 ±0.90	4.19 ±2.51	4.57 ±0.91	86.86	6.95	2.48	9.43	3.90
CTRL	90.40 ±0.95	2.42 ±0.44	1.08 ±0.20	2.77 ±0.52	5.70 ±0.30	91.55 ±0.66	2.90 ±0.54	0.87 ±0.14	3.77 ±0.62	4.68 ±0.80

[1]Values are means ± standard error of the mean.

[2]Results of unstimulated cultures were found to be significantly different from stimulated cultures for all phases of the cell cycle. For unstimulated cultures there was no significant differences for ±2-ME.

[3]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[4]For +2-ME, n=4, 3, 4, 3 & 4 and for -2-ME, n=3, 2, 3, 1 & 4, for ZnDF&LP, ZnDF, LP, ER and CTRL, respectively.

Table 7

Effects of dietary treatment and 2-mercaptoethanol treatment in vitro on the percentage of ConA-stimulated murine spleen cells in each phase of the cell cycle[1,2]

Treatment Group[3]	Culture Conditions									
	+2-ME					-2-ME				
	G_0	Early S	Late S	Total S	Mitosis	G_0	Early S	Late S	Total S	Mitosis
ZnDF&LP[4]	78.75^{A} ↑11.9%	9.51^{B} ↓29.8%	7.69^{B} ↓36.9%	17.21^{B} ↓33.1%	4.06 ↑12.2%	86.72 ↑0.1%	4.70 ↑6.3%	3.62 ↓7.4%	8.34 ↓0.1%	4.95^{B,C} ↓1.2%
ZnDF	79.79^{A} ↑13.4%	8.44^{B} ↓37.7%	7.38^{B} ↓39.4%	15.82^{B} ↓38.5%	4.44 ↑22.7%	87.83 ↑1.4%	4.73 ↑7.0%	2.66 ↓32.0%	7.38 ↓11.4%	4.80^{C} ↓4.2%
LP	80.03^{A} ↑13.7%	8.46^{B} ↓37.6%	7.32^{B} ↓39.9%	15.77^{B} ↓38.7%	4.13 ↑14.0%	86.95 ↑0.3%	3.81 ↓13.8%	2.68 ↓31.5%	6.50 ↓22.0%	6.56^{A} ↑30.9%
ER	76.36^{A,B} ↑8.52%	9.85^{A,B} ↓27.3%	9.07^{A,B} ↓25.5%	18.92^{B} ↓26.4%	4.72 ↑30.4%	84.16 ↓2.9%	5.14 ↑16.3%	4.34 ↑11.0%	9.48 ↑13.8%	6.37^{A,B} ↑27.1%
CTRL	70.36^{B}	13.55^{A}	12.18^{A}	25.72^{A}	3.62	86.66	4.42	3.91	8.33	5.01^{B,C}
Pooled SE[5]	5.36	3.21	2.87	5.41	0.99	2.37	1.73	1.27	2.61	0.99

[1]Values are means and percent increase or decrease from the CTRL for that culture condition. Different superscript letters indicate significant differences between means for a variable within a culture condition, as determined by Duncan's multiple range test.
[2]Significant main effects were stimulation and culture condition for all phases, except for M phase, where culture condition was not significant. When ANOVA was performed on stimulated samples only, a significant effect of diet was found for G_0, early S, late S, and total S phases for +2-ME samples. No significant diet effects were found for cells cultured without 2-ME.
[3]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.
[4]For +2-ME, n=6, 7, 5, 4 & 8 and for -2-ME, n=4, 6, 4, 3 & 7, for ZnDF&LP, ZnDF, LP, ER and CTRL, respectively
[5]To determine the standard error of the mean for a particular diet mean, divide the pooled standard error (SE) for a variable by the square root of n for that variable.

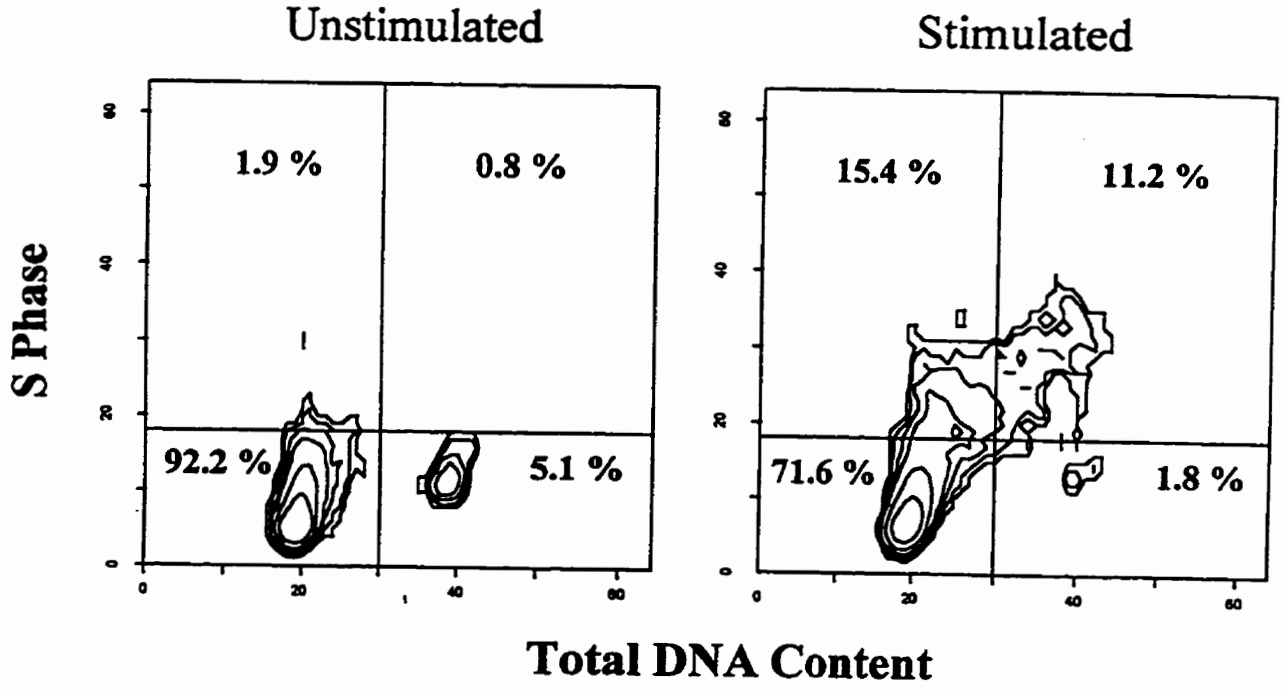
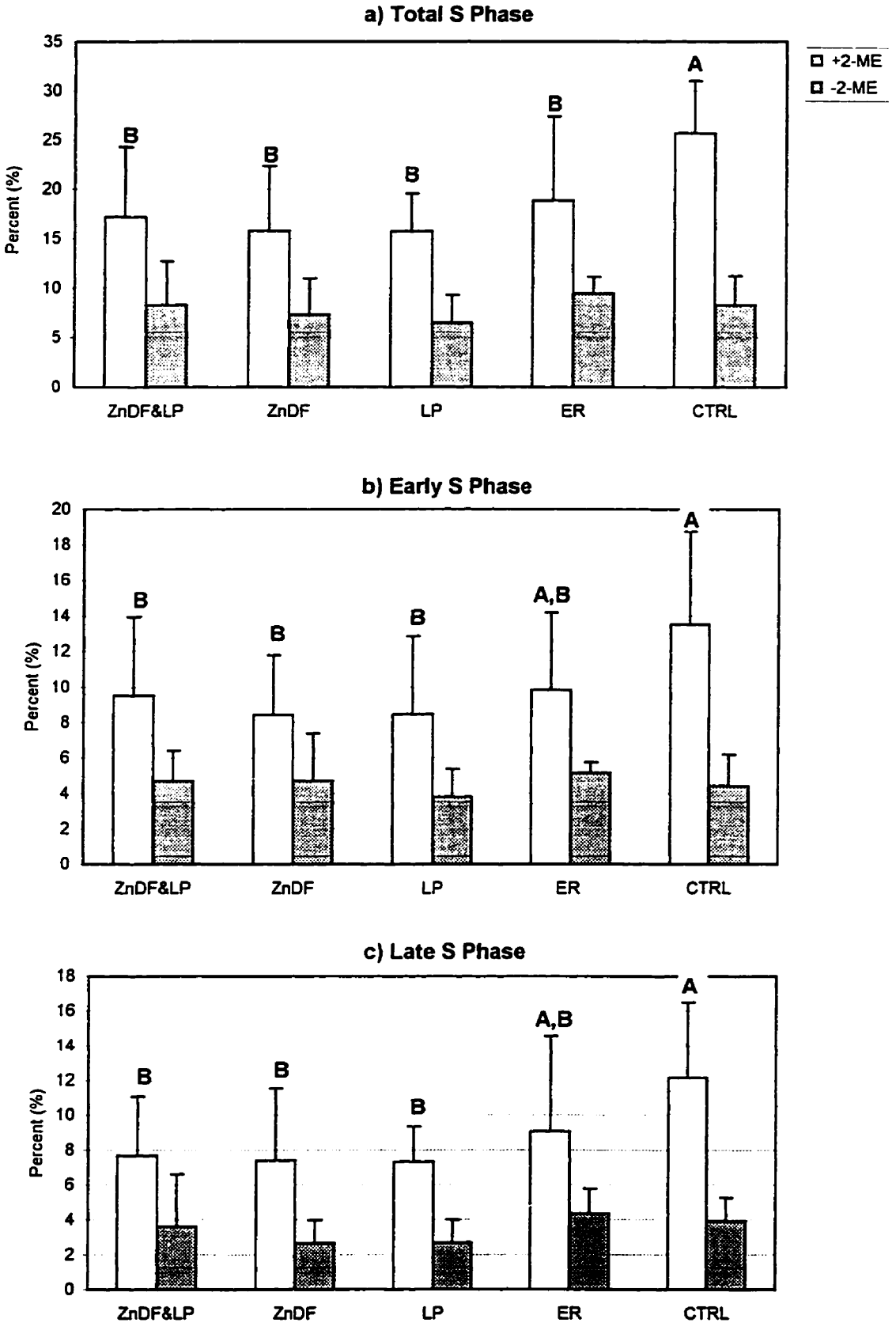


Figure 9. Results of cell cycle analysis by flow cytometry: Comparison of an unstimulated versus a ConcanavalinA-stimulated sample. Percentages in quadrants indicate the fraction of T-lymphocytes in each phase of the cell cycle. The lower left quadrant represents cell in G0 phase. The upper left quadrant represents cells in early S phase. The upper right quadrant represents cells in late S phase. The lower right quadrant represents cells in mitosis.


a) Total S Phase
Percent (%)
35
30
25
20
15
10
5
0
B
B
B
B
A
ZnDF&LP
ZnDF
LP
ER
CTRL
+2-ME
-2-ME
b) Early S Phase
Percent (%)
20
18
16
14
12
10
8
6
4
2
0
B
B
B
A,B
A
ZnDF&LP
ZnDF
LP
ER
CTRL
c) Late S Phase
Percent (%)
18
16
14
12
10
8
6
4
2
0
B
B
B
A,B
A
ZnDF&LP
ZnDF
LP
ER
CTRL

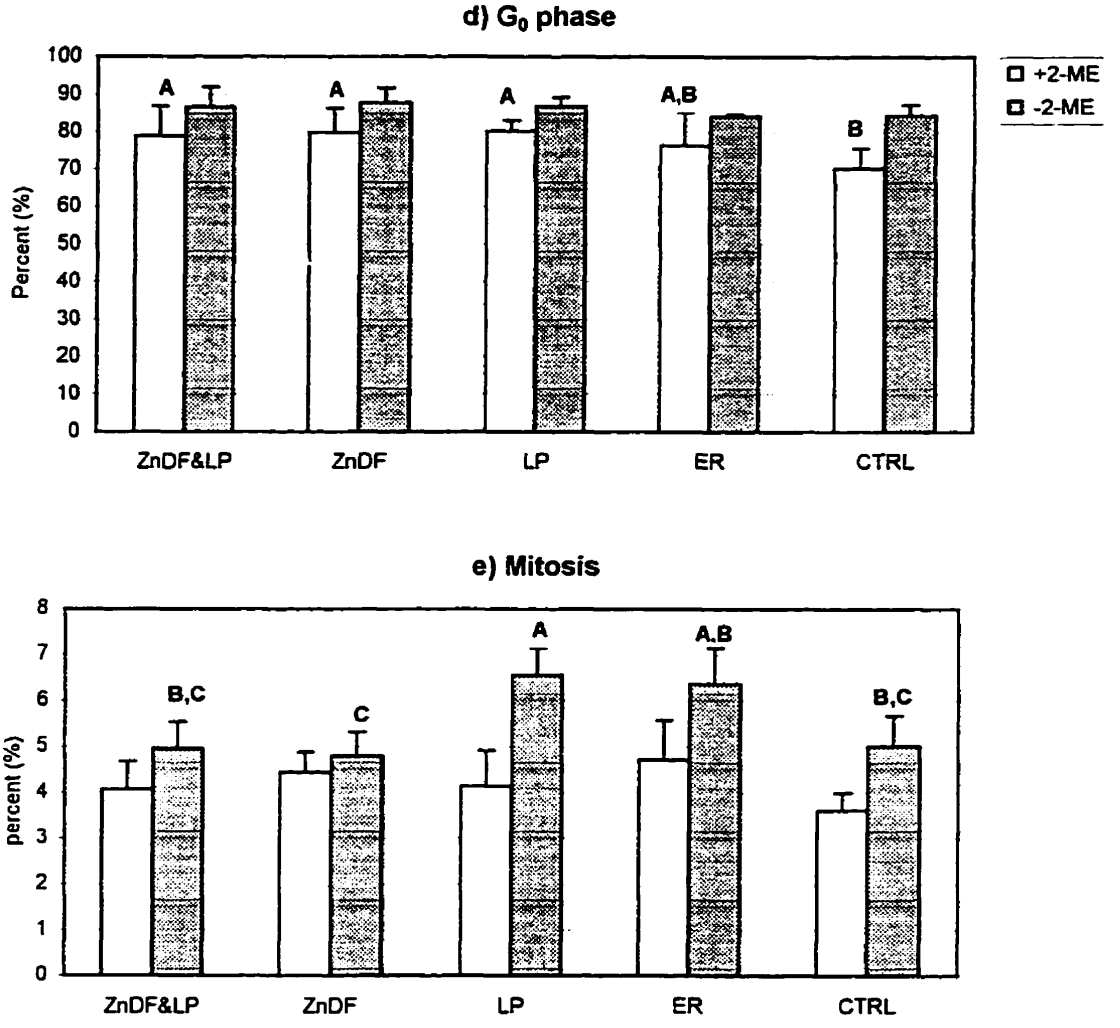


Figure 10a-e. Effects of dietary treatment and 2-mercaptoethanol (2-ME) treatment in vitro on the percentage of ConA-stimulated murine spleen cells in each phase of the cell cycle. Bars represent means ± the standard error of the mean. Upper case letters indicate significant differences between means for a variable within a culture condition, as determined by Duncan's multiple range test. Significant main effects were stimulation and culture condition for all phases, except for M phase, where culture condition was not significant. When ANOVA was performed on stimulated samples only, a significant effect of day was found for G_0, early S, late S and total S phases for +2-ME samples, and for G_0 and late S phases for -2-ME samples. A significant effect of diet was found for G_0, early S, late S and total S phases for +2-ME samples. No significant diet effects were found for cells cultured without 2-ME. ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control. For +2-ME, n=6, 7, 5, 4 & 8 and for -2-ME, n=4, 6, 4, 3 & 7, for ZnDF&LP, ZnDF, LP, ER and CTRL, respectively.

V. DISCUSSION

The objective of this thesis was to investigate the effects of dietary zinc deficiency and PEM on the immune system in adult mice, particularly regarding T-lymphocyte subpopulations and cell cycle progression. The most interesting finding was that dietary deficiency, whether a deficiency of a general nature, such as protein deficiency, or a deficiency of a more specific nature, such as deficiency of the micronutrient zinc, caused the inhibition of ConA-stimulated T-lymphocyte cell cycle progression. It appears that there is an interference in the transition of T-cells from the resting phase of the cell cycle into the DNA synthesis phase of the cell cycle. It was also confirmed that thiol status of the culture media is an important determinant of cell cycle progression for cells from mice of all dietary treatments. Among the dietary treatment groups, T-lymphocyte subpopulations were affected to some degree, with an increase in CD3+ cells in the LP and ER groups compared to the CTRL group, and a decrease in the CD4+ cells in the ZnDF&LP group compared to the CTRL group. However, no significant change in the CD4+/CD8+ ratio was observed among the diets. Zinc deficiency and PEM were found to adversely affect body weight, spleen weight and spleen cell numbers to varying degrees. These results will be discussed in more detail in the subsequent sections.

Effects of Zinc deficiency and PEM on body weight

It has previously been shown in our lab that the mouse models used in the experiments for this thesis are successful in inducing dietary zinc deficiency and

malnutrition syndromes (Lepage, 1997). Using this adult murine model allowed us to focus more clearly on the effects of the various dietary deficiencies on the immunological parameters of interest, without being influenced by confounding factors, such as growth and maturation, as in the case of the weanling models commonly used.

Experiment 1

By the end of the four week feeding trial, all four deficient groups had mean weights significantly lower than the CTRL group (Figure 7). The largest weight loss was observed in the ZnDF&LP group, which was not surprising, as it was expected that the combination of the two deficiencies would have the most detrimental effect. However, these results are in contrast to a previous study done in our lab in which the greatest weight loss was observed in the LP group (Lepage, 1997). Although the study by Lepage (1997) used the same diets, the same age and strain of mice, and the same housing conditions, the weights observed in the present study were considerably lower for all dietary groups, despite the fact that the baseline weights were almost the same. The differences in weights observed between these two studies may have been due to the fact that the animal housing unit was undergoing major renovations during this experiment. Unfortunately the investigators received no warning that the renovations were scheduled. The unusually high noise levels, smell of paint and disturbance caused by shuffling mice to different rooms almost certainly increased the stress level of the mice in the study. This additional stress may have served as a contributing factor for the lower body weights observed. The fact that the ZnDF&LP group experienced the most weight loss, 3 g more than that observed by Lepage (1997), may suggest that the combined dietary deficiency

may be tolerated under normal conditions, but that these mice are less able to tolerate additional stress levels.

Zinc deficiency alone has been shown by other researchers to cause decreased body weights. A study by King & Fraker (1991), in which six-week old A/J female mice were fed a zinc deficient diet (0.8 ppm Zn) for 30 days, found that zinc-deficient mice weighed 28-30% less than mice fed a zinc-adequate diet. This is a much greater weight loss than observed in the present study, in which ZnDF mice weighed 11% less than CTRL mice. The main difference between the two studies is the age of the mice, which most likely accounts for the difference in the observed weight loss. King & Fraker (1991) used six-week old mice which are growing at a much more rapid rate than the 4 month old mice used in the current study. Zinc is known to be required for growth, therefore adolescent mice exhibiting high growth rates will be more affected by a zinc deficient diet than adult mice whose growth rate has tapered off. Adult mice were chosen for the present study to eliminate the complicating factor of rapid growth and to focus more clearly on the effects of zinc deficiency on weight.

Other studies have shown that a low protein diet also affects weight gain. Taylor and colleagues (1997) found that the mean body weights of 3-4 month old mice fed a 0.5% protein diet were 30% less than the mean weight of the control group at the end of a four week feeding trial. This is a much larger weight difference than the 17% difference between the LP group and the CTRL group observed in the present study. The difference in the weight loss of the LP group that occurred may be due to the fact that the low-protein diet used by Taylor and colleagues provided only 0.5% protein, compared to the 2% protein diet provided in the present study. The more severe protein restriction

would be expected to cause a greater weight loss. In general, it appears that a low protein diet has a greater effect on body weight than does zinc deficiency.

Another observation from this study was the dramatic weight loss that occurred during the first week the mice were on the treatment diets (an average of 2.0-2.5 g). This was observed in all treatment groups, so was more likely a reflection of the adaptation of the mice to the different cages (ie. hanging cages with wire mesh bottoms compared to shoebox cages bedded with wood shavings), the different form of feed (ie. powdered diet compared to pelleted chow) and the different way in which the feed was presented to them (ie. glass jars compared to pellets provided in overhanging mesh container). After this first week of adaptation, the trend in the weights followed a pattern that was more reflective of the diet the mouse was receiving.

Experiment 2

By the end of the 4 week feeding trial, all four deficient groups had weights significantly lower than the CTRL group. By the third week, the ZnDF&LP and LP groups had significantly lower weights than the ZnDF group (Figure 8 and Appendix B). The LP group experienced the most dramatic weight loss (27% decrease) compared to the CTRL group.

The weights observed in this experiment were more similar to that observed by Lepage (1997), who found that the LP group also experienced the greatest weight loss (17%) compared to the CTRL group. The weights recorded in Experiment 2 are more likely to be similar to those recorded by Lepage as the renovations taking place during

Experiment 1 had been completed, so the mice in Experiment 2 were not exposed to any additional stress factors.

Due to the large decrease in weight observed in Experiment 1 during the first week on the treatment diets, Experiment 2 included an acclimatization period during which the mice were fed powdered CTRL diet in hanging cages for one week and then transferred to clean hanging cages and fed their respective treatment diets. In general, by the end of the 4 week feeding trial, the mean weights of groups in Experiment 2 were higher than the mean weights in Experiment 1, except for the LP group which had a lower mean weight in Experiment 2.

Assessment of Immunological Parameters

Experiment 1

Both PEM and zinc deficiency have previously been documented to affect the immune system by causing a decreased spleen weight and also decreased spleen cell numbers (Fraker et al, 1977; Fraker et al, 1978; Woodward & Miller, 1991). However, despite the fact that these two deficiencies are closely linked, few studies have looked at the effect of these two deficiencies in combination.

Spleen weights of mice from all four deficient groups were significantly lower than the spleen weights of the baseline and CTRL groups, regardless of whether it was expressed in absolute terms or relative to body weight (Table 3 and Appendix D). It is interesting to note that although the combination of a zinc deficient and a low protein diet resulted in the lowest average spleen weight, this was not significantly different than the spleen weights of the mice fed either the zinc deficient or the low protein diet.

Comparison of the average spleen cell numbers of the animals in the deficient groups to the spleen cell numbers of the CTRL group indicates that the combined deficiency of both zinc and protein had the most detrimental effect on spleen cell numbers, as it was the only group that had a significantly lower spleen cell count. The other three deficient groups did have reduced spleen cell numbers compared to the CTRL group, but the differences were not significant.

Experiment 2

Average spleen weights and spleen cell numbers for the ZnDF, ER and CTRL groups from Experiment 2 were very similar to those observed in Experiment 1 (Table 5 and Appendix F). The main differences between the two experiments was in the ZnDF&LP and LP groups. In Experiment 1, the ZnDF&LP group had the lowest spleen weight and was the only group to have a splenocyte count significantly lower than the CTRL group. However, in Experiment 2, the LP group had the lowest spleen weight and was the only group to have a splenocyte count significantly lower than the CTRL group. Lepage (1997) found that the LP diet had a greater effect on spleen weight and splenocyte count than did the combined deficiency of zinc and protein. These observations are more similar to those from Experiment 2.

The differences between Experiment 1 and Experiment 2, and also between Experiment 1 and Lepage (1997) may be attributed to the renovations taking place in the animal housing unit during Experiment 1. It is possible that the mice receiving the combined zinc deficient and low protein diet have reduced ability to handle increased

stress levels, which may exacerbate the adverse effects of their deficient diets on body weight and immune capacity.

There is considerable research showing that zinc and protein deficient diets cause a decrease in spleen weight and a decrease in spleen cell numbers, although there are few studies that look at these two deficiencies in conjunction. Bises and coworkers (1987) studied the effects of dietary zinc deficiency and protein deficiency in rats, but did not feed a combined zinc and protein deficient diet. They found that the spleen weight was drastically reduced in rats fed either a zinc deficient or a protein deficient diet (4% or <1% protein). Splenocyte numbers were also significantly reduced with all three deficient diets. The most dramatic decrease in splenocyte numbers was observed in the severely protein deficient (<1%) group. This corresponds with the results of Experiment 2, in which the LP group had the lowest spleen cell count.

Studies with mice show similar results. Cook-Mills and Fraker (1993) found that adult female mice fed a zinc restricted diet (0.8 μg Zn/g) had significantly reduced spleen cell numbers compared to the control group fed a zinc adequate diet. Woodward and Miller (1991) reported decreased splenocyte numbers for male weanling mice fed a 0.5% protein diet. Petro (1985) also found that female weanling mice had significantly reduced splenocyte numbers when fed a 4% or 2% protein diet for five weeks.

Evaluation of Zinc Status

Experiment 1 and 2

It can be concluded that serum zinc concentrations are adversely affected by dietary zinc deficiency and PEM. In both Experiment 1 and Experiment 2, mice in the

two zinc deficient groups (ie ZnDF&LP and ZnDF) had lower serum zinc concentrations (between 44.9% and 54.4%) than the CTRL group (Table 2 and Appendix C). The pattern of serum zinc concentrations and the magnitude of reduction seen in both experiments is similar to that observed previously in our lab by Lepage (1997) and those observed by Cook-Mills & Fraker (1993) in young adult mice.

A decreased serum concentration of about 50% has been shown to occur within hours of initiation of feeding a zinc deficient diet and lasts for the duration of the dietary zinc deficiency (Cousins, 1989). Due to the fact that this effect of zinc deficiency has been well documented, reduced plasma zinc concentrations have become "a recognized standard of status assessment in uncomplicated zinc deficiency experiments with laboratory animals maintained under carefully regulated environmental and dietary conditions" (Cousins, 1989, p.81). Serum zinc concentration may be measured instead of plasma zinc, although slightly higher zinc concentrations are present in serum.

Experimental zinc deficiency in human subjects has also been shown to produce a reduction in plasma zinc concentrations. In a clinical setting, patients receiving total parenteral nutrition without zinc supplementation have also been shown to experience a decreased plasma zinc concentration (Cousins, 1989).

Interestingly enough, the LP group also experienced a reduction in serum zinc compared to the CTRL group in both experiments, and this difference was significant in Experiment 2. This result is not entirely unlikely, due to the fact that severe malnutrition has previously been documented to cause depressed serum zinc status (Cousins, 1989), although the metabolic basis for this phenomena is not well understood. Filteau and Woodward (1982) have also demonstrated that the decreased serum zinc concentration

observed in weanling mice fed a protein deficient diet (1.7%) cannot be restored with dietary zinc supplementation (200 μg/g diet), suggesting that the depressed serum zinc levels are due to some aspect of the metabolic changes induced by the protein deficiency. It is also interesting to note that the combination of a zinc deficient and a low protein diet in the current study did not exacerbate the depression in serum zinc concentration in the ZnDF&LP group compared to zinc deficiency alone.

It has been suggested that the hypozincaemia observed in PEM may be due to depressed plasma albumin levels, since albumin is the major carrier for zinc in the blood. However, Golden and Golden (1979), found that malnourished children had depressed plasma zinc concentrations, but their plasma albumin concentrations were normal. Lepage (1997) also found that serum zinc concentrations did not correspond with serum albumin concentrations. It has also been suggested that the zinc deficiency associated with PEM may be due to a decreased absorption of zinc as a result of the change in the intestinal mucosa that occurs in conjunction with malnutrition (Atalay et al, 1989).

Although femur Zn was lower (12.4-18.5%) in the deficient groups when compared to the CTRL group, this was not statistically significant (Table 2 and Appendix C). No differences were observed among the treatment groups regarding spleen zinc concentrations. Femur zinc is not as sensitive an indicator of zinc status over the short term as is serum zinc, because the zinc in bone turns over at a slower rate than does the zinc in the blood. For this reason it is more difficult to detect changes in femur zinc with short term zinc deficiency. Larger differences in femur zinc may have been observed if the feeding trial had been extended beyond the 4 week period. Also, there is more chance of error when measuring femur zinc, as extensive sample preparation is involved,

which increases the opportunity for sample contamination. A similar study in our lab by Lepage (1997) showed a significant reduction in femur zinc in the ZnDF&LP and ZnDF groups compared to the CTRL group (14.8% and 21.1% respectively), and that the pattern of femur zinc concentration was closely related to the pattern of serum zinc concentration.

T-Lymphocyte Subpopulations

Although changes in the T-lymphocyte subpopulations were observed, a significant alteration in the ratio of CD4+/CD8+ cells did not occur in deficient mice (Table 4 and Appendix E). The ratio of CD4+/CD8+ T-lymphocytes is often used as an indication of immune function as an imbalance in the ratio of T-helper (CD4+) to T-cytotoxic/suppressor (CD8+) cells results in compromised immune function.

The percentage of T-lymphocytes expressing CD3 was higher in all four deficient groups compared to the baseline and CTRL groups. This difference was significantly higher for the LP and ER groups. The percentage of CD3+ T-cells represents the percentage of mononuclear cells that were removed from the initial cell preparation procedure as part of the interphase layer, and then later gated on as lymphocytes by the flow cytometer and identified by the staining of CD3 with a flourescent-conjugated anti-CD3 antibody. There are a number of possible explanations for the differences in the percentage of CD3+ T-cells among the dietary groups that may not necessarily be attributed to the effect of diet. For example, the percentage of CD3+ cells identified may be influenced by the separation procedure, depending on the efficiency of the removal of the interphase layer. As well, there may be changes in other cell population numbers

which may influence the percentage of CD3+ T-cells. For example, in the baseline and CTRL groups, which had a lower percentage of CD3+ cells, perhaps there was a larger proportion of B-lymphocytes, or other immune cells, that may have been included in the lymphocyte gate but do not express CD3. The presence of other cells within the lymphocyte gate will lower the percentage of CD3+ T-cells. It may be important for future studies regarding T-lymphocyte subpopulations to also determine the percentage of B-lymphocytes present. King and Fraker (1991) have also studied the phenotypic distribution of splenic lymphocytes in zinc deficient adult mice and have also reported an increase in T-cells in the restricted-zinc-adequate group (similar to the LP or the ER group in the current study), compared to the control group.

A significant difference was observed in the percentage of CD3+ cells expressing CD4. The ZnDF&LP group had a significantly lower percentage of CD4+ cells compared to all groups other than the ZnDF group. The ZnDF group had the next lowest percentage of CD4+ cells, which was significantly lower than the baseline group, but not the CTRL group. There were no significant differences among the groups regarding the percentage of CD3+CD8+ cells, nor were there significant differences noted among the groups regarding the CD4+/CD8+ ratio.

These results are quite different from the results of papers previously published. Taylor and colleagues (1997) found that adult mice fed a 0.5% protein diet for 4 or 6 weeks had an increased percentage of CD4+ T-lymphocytes compared to controls, while the percentage of CD8+ remained unchanged. There was also a significant increase in the CD4/CD8 ratio. Perhaps the more severe protein restriction used in that study (0.5%) compared to the 2% protein diet used in this experiment may be a factor in explaining the

differences in results. Lee and Woodward (1996) also reported an increase in the percentage of CD4+ cells and an increase in the CD4+/CD8+ ratio of spleen cells from weanling mice fed a 0.6% protein diet. Both of these studies looked at the percentage of CD4+ and CD8+ cells among splenocytes and not only as a percentage of CD3+ cells, so the absolute percentages cannot be compared to the present results.

Results of previous studies investigating the effects of dietary zinc deficiency on T-lymphocyte subsets also differ from the results of the current study. King and Fraker (1991) found that severely zinc deficient weanling mice (those exhibiting a significant degree of parakeratosis and weighing 65.3 $\pm$ 3% of the body weight of the zinc adequate mice) demonstrated a 20% increase in the overall ratio of CD4+/CD8+, while the marginally zinc deficient mice (weighing 70.6 $\pm$ 0.7% of the average body weight of the zinc adequate group and exhibiting only modest degrees of parakeratosis) did not have a significantly increased ratio of CD4+/CD8+ compared to the zinc adequate group. Similarly to the two experiments of protein deficiency described above, the increased ratio of CD4+/CD8+ was due to an increase in the percentage of CD4+ cells rather than a change in the CD8+ cells. Two studies investigating the effect of zinc deficient diets on T-lymphocyte subpopulations in rats did not find any significant alteration in the T-lymphocyte subsets of zinc deficient rats (Bises et al, 1987; Dowd et al, 1986).

Since reference has only been made to two subpopulations of T-lymphocytes throughout this thesis, it may seem logical that when the percentage of CD3+ T-cells expressing either CD4 or CD8 receptors is added up, that the percentages should equal 100%. However, this is not the case, as indicated by the totals for CD4+ and CD8+ in Table 4. In fact, there are four different subsets of T-lymphocytes: a) CD4+CD8- (T-

helper cells), b) CD4-CD8+ (T-cytotoxic/suppressor cells), c) CD4-CD8- ("double-negative" cells), and d) CD4+CD8+ ("double positive" cells). During the maturation of T-lymphocytes, immature T-cells in the cortex of the thymus originally express neither CD4 nor CD8 (ie. "double-negative" cells). A fraction of these immature cells then differentiate to become "double-positive" cells, expressing both CD4 and CD8. Following this stage, the cells travel to the medulla of the thymus where they undergo a process called "positive/negative selection", during which time either CD8 or CD4 is down-regulated producing CD4+CD8- T-lymphocytes or CD4-CD8+ T-lymphocytes respectively (Abbas et al, 1991; Godfrey & Zlotnik, 1993).

Normally, the adult mouse thymus has been shown to contain about 5% of the T-lymphocytes in the immature "double-negative" form (Abbas et al, 1991). Since the results presented in Table 4 indicate only CD4+ and CD8+ T-cells, the difference of the total of these two from 100% may be interpreted as the percentage of immature double negative T-lymphocytes present in the cell preparation. That is, the larger the deviation from 100%, the greater the number of immature T-lymphocytes present. Thus, an increased number of immature cells may be partially responsible for the decreased immune function observed in mice with PEM and zinc deficiency, as immature "double-negative" cells have limited capacity to function as mature T-lymphocytes (Abbas et al, 1991).

As indicated in Table 4 and Appendix E, the total percentage of CD4+ and CD8+ T-cells was lower for all deficient groups compared to the baseline and CTRL groups, indicating that the mice fed the deficient diets may have an increased number of immature T-lymphocytes. The ZnDF&LP group had a significantly lower total

percentage of CD4+ and CD8+ T-cells compared to all the other groups, except the ZnDF group, while the ZnDF, LP and ER groups all had significantly lower total CD4+ and CD8+ than the baseline and CTRL groups.

Related to this is the finding that zinc deficiency may induce apoptosis of thymocytes in mice (Telford & Fraker, 1995). This cell culture experiment found that low zinc concentrations (80-200 μM) in the culture media could induce apoptosis in approximately 30-40% of mouse thymocytes during incubation. Thus, the decrease seen in the total percentage of CD4+ and CD8+ mature lymphocytes in the deficient groups may be the result of an increased rate of apoptosis of mature T-lymphocytes, resulting in a proportionately larger percentage of immature T-lymphocytes.

Cell Cycle Analysis

Cell cycle analysis by BrdU incorporation and flow cytometry analysis confirmed previous findings that 2-ME is essential for proliferation of murine T-lymphocytes in culture. Regardless of the source of T-lymphocytes (ie. from mice fed deficient diets or mice fed a nutritionally complete diet), cells cultured in the presence of a mitogen but without 2-ME exhibited significantly decreased proliferation. This was quantitated as an increase in the percentage of cells in the G_0 phase of the cell cycle and a decrease in the percentage of cells in the S phase of the cell cycle (Figure 10).

When Con-A stimulated T-lymphocytes were cultured with 2-ME, decreased T-lymphocyte proliferation was seen in cells from deficient mice compared to cells from CTRL mice (Figure 10). The decreased proliferation was indicated by an increase in the percentage of cells in the G_0, or resting phase of the cell cycle, and a concomitant

decrease in the percentage of cells in the S phase, or DNA synthesis phase, of the cell cycle. Thus, it appeared that a compromised nutritional state, regardless of whether the deficiency is due to a macronutrient deficiency (ie. protein) or a micronutrient deficiency (ie. zinc), decreased T-lymphocyte proliferation in thiol-supplemented media.

Taylor and colleagues (1997) also found that 2-ME in culture enhances proliferation of murine T-lymphocytes. However, they found that when the anti-CD3-stimulated splenocytes were cultured without 2-ME, splenocytes from mice fed a low-protein diet (0.5%) had an increased proliferative response compared to splenocytes from mice fed the control diet (15% protein). This differs from the findings of the present study, in which diet had no effect when cells were cultured in media lacking 2-ME. Perhaps no differences were seen among diet groups in the present study, due to the fact that the protein restriction was not as severe (2%) compared to the 0.5% restriction used by Taylor and coworkers (1997). There are numerous other differences between the two studies that may account for the differences in the results. Taylor and colleagues (1997) used a different technique for analyzing cell proliferation. The technique involved the incorporation of BrdU into the DNA of proliferating cells in culture, after which the cells are stained with Hoechst, which identifies the percentage of proliferating cells. Although both their experiment and the present experiment used BrdU incorporation, the length of time that the cultured cells were exposed to BrdU varies from 72 hours in their study to 4 hours in the current study. This may affect the interpretation of the results and may account for some of the differences seen. The length of culture also varies from 72 hours in their study to 48 hours in the current study. Another difference is the method of cell stimulation, Taylor and coworkers (1997) stimulated T-lymphocytes by plating the

culture wells with anti-CD3, compared to the use of ConA to stimulate T-cells in the current study. Petro (1985) also found that splenocytes from weanling mice fed either a 4% or a 2% protein diet for five weeks had an increased in vitro mitogenic response compared to splenocytes from control mice.

When the in vitro function of 2-ME is considered, it is not surprising that cells cultured without 2-ME would have sub-optimal proliferative responses to a mitogen. 2-ME has a fundamental role in increasing cysteine transport into cells (Ishii et al, 1981). Cysteine is the rate-limiting amino acid required for intracellular glutathione synthesis, and glutathione has been shown to be important for the proliferative capacity of T-lymphocytes. Fidelus and colleagues (1987) showed that modulation of intracellular glutathione concentration in murine lymphocytes can alter cellular activation and proliferation. Cells cultured with 2-ME in the presence of ConA were found to have significantly higher intracellular glutathione content than cells cultured without 2-ME. Lymphocytes cultured in the presence of 2-ME and stimulated with ConA, were analyzed by a flow cytometric method using acridine-orange. It was found that not only does 2-ME augment the number of stimulated cells that enter S phase, but it may also act as a weak mitogen by itself.

Thiol-supplementation of culture media with 2-ME may increase the proliferative response of lymphocytes by additional mechanisms. 2-ME has been shown to increase the intracellular glutathione concentration of cells in culture, and glutathione is thought to be important in mediating the redox-status of cells (Droge et al, 1994). Many of the genes involved in cell proliferation are regulated by transcription factors that are sensitive to thiol redox status for DNA binding activity. An example is nuclear

transcription factor κB (NFκB) which is involved in the inducible transcription of several immunologically important genes, such as the interleukin-2 receptor α-chain and major histocompatibility complex antigens. NFκB has been shown to be sensitive to redox state, as it is inhibited in vitro by oxidation and reactivated by thiols such as 2-ME (Droge et al, 1994).

Several signal transducing proteins which function as tyrosine kinases in the T-lymphocyte signal transduction pathway (ie. $p56^{lck}$) are also known to be redox-sensitive (Droge et al, 1994). The formation of the mixed disulfide 2-ME and cysteine may act directly on these signal transducing proteins, thereby affecting T-lymphocyte signal transduction and ultimately cell proliferation (Droge et al, 1994).

Studies using cell lines support the hypothesis that zinc is required for cell proliferation (Chester et al, 1993; Chesters & Boyne, 1991), however, studies regarding the effects of dietary zinc deficiency on cell proliferation are not conclusive. Kramer (1984) indicated that although dietary zinc deficiency caused major changes in total-body and organ growth, it had little effect on the in vitro mitogenic-induced proliferation of lymphocytes. Other studies have also shown that cells from zinc deficient rodents maintain their ability to proliferate in culture (Cook-Mills & Fraker, 1993; Mengheri et al, 1988). However, Moulder & Steward (1989) showed that zinc deficient mice had reduced T-lymphocyte proliferation in response to ConA-stimulation.

The current study shows that malnutrition, caused by either a deficiency of zinc or protein, affects the ability of lymphocytes to proliferate in vitro. There does not appear to be an affect associated specifically with deficiency of dietary zinc, as similar decreases in proliferation were observed in lymphocytes from mice in all four deficient groups.

However, this does not rule out the possibility that zinc may play a role in T-lymphocyte proliferation. Future research may address this question by conducting a study with the same protocol as for Experiment 2, with the use of zinc chelators to manipulate the zinc content of the media. This would allow the investigators to determine if there is a specific role for zinc in cell cycle progression.

In addition, future research regarding the effect of dietary zinc deficiency and PEM on the level of cyclin-dependent protein kinases (CDKs) may provide important information about the decrease in cell cycle progression in the deficient animals. CDKs have emerged in recent years as key regulators of cell cycle progression (Reddy, 1994). Changes in the level, as well as the activity of CDKs, and their association with cyclins involved in the progression of cells from one phase to another. Numerous cyclins, in association with CDKs, seem to function in the transition from G_1 to S phase. Western blotting techniques may be used to determine the levels of CDKs.

VI. CONCLUSIONS

GENERAL CONCLUSIONS:

- Dietary deficiencies of zinc and/or protein resulted in decreased body weight, decreased spleen weight and decreased serum zinc concentrations.
- Only the LP diet resulted in significantly decreased spleen cell numbers.

CONCLUSIONS REGARDING T-LYMPHOCYTE SUBPOPULATIONS:

- All four deficient groups had a higher percentage of CD3+ cells than the baseline and CTRL groups, although only the LP and ER groups were significantly higher.
- Only the ZnDF&LP diet resulted in a significantly decreased percentage of CD4+ T-lymphocytes.
- There were no significant differences in the percentage of CD8+ T-lymphocytes or in the CD4+/CD8+ ratio.
- All four deficient groups had a significantly lower total percentage of CD4+ and CD8+ compared to the baseline and CTRL groups, which may indicate that these mice have increased percentages of immature "double-negative" T-cells compared to CTRL mice.

CELL CYCLE PROGRESSION:

- Dietary deficiencies of zinc and/or protein resulted in an increased number of cells in the G_0 phase of the cell cycle and a decreased number of cells in the S phase of the cell cycle.
- 2-ME in vitro is required for the cell cycle progression of murine T-lymphocytes and for the demonstration of decreased proliferative capability of T-lymphocytes in dietary deficient mice.

FUTURE RESEARCH:

- More research is needed regarding the specific molecular mechanism by which zinc deficiency affects the T-lymphocyte signal transduction pathway.
- More research is needed to investigate the effects on cell cycle progression of adding zinc to the media of cultured splenocytes from mice fed diets deficient in zinc and/or protein.
- Improvements need to be made in the ability to qualitatively assess the zinc status of patients in a clinical situation, so that zinc deficiency will not be misdiagnosed. Zinc deficiency is often the underlying cause of immuno-deficiency or other disease complications, although its role in the disease is often not recognized.

VII. REFERENCES

Abbas, A.K., Lichtman, A.H. & Pober, J.S. (1991) Cellular and molecular immunology (Wonsiewicz, M.J., ed.), pp.5-8,177-180. W.B. Saunders Company, Philadelphia, PA.

Abbott, W.C., Tayek, J.A., Bistrian, B.R., Maki, T., Ainsley, B.M., Reid, L.A. & Blackburn, G.L. (1986) The effect of nutritional support on T-lymphocyte subpopulations in protein calorie malnutrition. J. Amer. Col. Nutr. 5: 577-584.

Aggett, P.J. & Comerford, J.G. (1995) Zinc and human health. Nutr. Rev. 53: S16-S22.

Anon. (1983) Plasma levels of zinc in protein-calorie malnutrition and after nutritional rehabilitation. Nutr. Rev. 41: 209-210.

Atalay, Y., Arcasoy, A. & Kurkcuoglu, M. (1989) Oral plasma zinc tolerance test in patients with protein energy malnutrition. Arch. Dis. Child. 64: 1608-1611.

Baker, D.H. (1992) Cellular antioxidant status and human immunodeficiency virus replication. Nutr. Rev. 50: 15-18.

Beach, R.S., Gershwin, M.E., Makishma, R.K. & Hurley, L.S. (1980) Impaired immunologic ontogeny in postnatal zinc deprivation. J. Nutr. 110: 805-815.

Bellomo, G., Vairetti, M., Stivala, L., Mirabelli, F., Richelmi, P. & Orrenius, S. (1992) Demonstration of nuclear compartmentalization of glutathione in hepatocytes. Proc. Natl. Acad. Sci. USA. 89: 4412-4416.

Bettger, W.J. & O'Dell, B.L. (1993) Physiological role of zinc in the plasma membrane of mammalian cells. J. Nutr. Biochem. 4: 194-207.

Bhaskaram, C. & Reddy, V. (1974) Cell mediated immunity in calorie malnutrition. Env. Child Health. Dec: 284-286.

Bises, G., Mengheri, E. & Gaetani, S. (1987) T-lymphocyte subsets in zinc deficient and in protein malnourished rats. Nutr. Rep. Int. 36: 1371-1378.

Bray, T.M. & Taylor, C.G. (1994) Enhancement of tissue glutathione for antioxidant and immune functions in malnutrition. Biochem. Pharmacol. 47: 2113-2123.

Canadian Council in Animal Care. (1993) Guide to the use and care of experimental animals. Volume 1. 2nd Edition. Bradda Printing Services Inc., Ottawa, Ontario.

Carlomagno, M.A. & 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.

Chandra, R.K. (1991) 1990 McCullom award lecture. Nutrition and immunity: lessons from the past and new insights into the future. Am. J. Clin. Nutr. 53: 1087-1101.

Chandra, R.K. (1983) The nutrition-immunity-infection nexis: The enumeration and functional assessment of lymphocyte subsets in nutritional deficiency. Nutr. Res. 3: 605-615.

Chandra, R.K. & Au, B. (1980) Single nutrient deficiency and cell-mediated immune responses: Zinc. Am. J. Clin. Nutr. 33: 736-738.

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

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

Chesters, J.K., Petrie, L & Vint, H. (1989) Specificity and timing of the Zn^{2+} requiement for DNA synthesis by 3T3 cells. Exp. Cell Res. 184: 499-508.

Clegg, M.S., Kenn, C.L., Lonnerdal, B. & Hurley, L.S. (1981) Influence of ashing techniques on the analysis of trace elements in animal tissues. I. Wet ashing. Biol. Trace Elem. Res. 3: 107-115.

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

Cousins, R.J. (1996) Zinc. In: Present Knowledge in Nutrition, 7th edition (Ekhard, E., Ziegler, E. & Filer, L.J., ed). pp. 293-306. ILSI Press, Washington, DC.

Cousins, R.J. (1989) Systemic transport of zinc. In: Zinc in Human Biology (Mills, C.F., ed). pp. 81-83. Springer-Verlag, NY.

Cunnane, S.C. (1988) Zinc: Clinical and Biochemical Significance. pp. 11-12, 31-36. CRC Press, Boca Ranton, FL.

DePasquale-Jardieu, P. & 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.

Deneke, S.M. & Fanburg, B.L. (1989) Regulation of cellular glutathione. Am. Physiol. Soc. L163-L173.

Dolbeare, F., Gratzner, H., Pallavicini, M.G., & Gray, J.W. (1983) Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc. Natl. Acad. Sci. USA. 80: 5573-5577.

Dolbeare, F., Kuo, W.L., Beisker, W., Vanderlaan, M., & Gray, J.W. (1990) Using monoclonal antibodies in bromodeoxyuridine-DNA analysis. Meth. Cell Biol. 33: 207-216.

Dolbeare, F. & Gray, J.W. (1988) Cytometry. 9: 631-635.

Dowd, P.S., Kelleher, J. & Goillou, P.J. (1986) T-lymphocyte subsets and interleukin-2 promotion in zinc deficient rats. Brit. J. Nutr. 55: 56-69.

Droge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, H.P., Roth, S. and Gmunder, H. (1994) Functions of glutathione and glutathione disulfide in immunology and immunopathology. FASEB J. 8: 1131-1138.

Eck, H.P., Gmunder, H., Hartmann, M., Petzoldt, D., Daniel, V. & Droge, W. (1989) Low concentrations of acid-soluble thiol (cysteine) in the blood plasma of HIV-1-infected patients. Biol. Chem. Hoppe-Seyler. 370: 101-108.

Evans, R.M. & Hollenberg, S.M. (1988) Zinc fingers: gilt by association. Cell. 52: 1-3.

Fernandes, G., Nair, M., Onoe, K., Tanaka, T., Floyd, R. & Good, R.A. (1979) Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice. Proc. Natl. Acad. Sci. USA. 76: 457-461.

Fidelus, R.K., Ginouves, P., Lawrence, D. and Tsan, M.F. (1987) Modulation of intracellular glutathione concentration alters lymphocyte activation and proliferation. Exp. Cell Res. 170: 269-275.

Filteau, S.M. & Woodward, B. (1982) The effect of severe protein deficiency on serum zinc concentrations of mice fed a requirement level or a very high level of dietary zinc. J. Nutr. 112: 1974-1977.

Fraker, P.J., Caruso, R. & 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., DePasquale-Jardieu, P., Zwickl, C.M. & Luecke, 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. & Leucke, R.W. (1977) Effect of zinc deficiency on the immune response of young adult A/J mouse. J. Nutr. 107: 1889-1895.

Gibson, R.S. (1989) Sub-optimal zinc status during infancy and childhood. NIN Rev. 11: 1-3.

Gibson, R.S., Vanderkooy, P.D.S., MacDonald, A.C., Goldman, A., Ryan, B.A. & Berry, M. (1989) A growth-limiting, mild zinc-deficiency syndrome in some southern Ontario boys with low height percentiles. Am. J. Clin. Nutr. 49: 1266-1273.

Godfrey, D.I. & Zlotnik, A. (1993) Control points in early T-cell development.

Golden, B.E. & Golden, M.H. (1979) Plasma zinc and the clinical features of malnutrition. Am. J. Clin. Nutr. 32: 2490-2494.

Gross, R.L., Osdin, N., Fong, L. & Newberne, P.M. (1979) I. Depressed immunological function in zinc-deprived rats as measured by mitogen response of spleen, thymus and peripheral blood. Am. J. Clin. Nutr. 32: 1260-1265.

Hamilos, D.L., Mascali, J.J. & Wedner, H.J. (1991) The role of glutathione in lymphocyte activation - II. Effects of buthionine sulfoximine and 2-cyclohexene-1-one on early and late activation events. Int. J. Immunopharmac. 13: 75-90.

Hansen, M.A., Fernandes, G. & Good, R.A. (1982) Nutrition and immunity: The influence of diet on autoimmunity and the role of zinc in the immune response. Ann. Rev. Nutr. 2: 151-177.

Hemalatha, P., Bhaskaram, P. & Khan, M.M. (1993) Role of plasma zinc supplementation in the rehabilitation of severely malnourished children. Eur. J. Clin. Nutr. 47: 395-399.

Heywood, P.F. & Marks, G.C. (1993) Nutrition and health in South-East Asia. Med. J. Australia. 159: 133-137.

Ishii, T., Sugita, Y. & Bannai, S. (1987) Regulation of glutathione levels in mouse spleen lymphocytes by transport of cysteine. J. Cell. Physiol. 133: 330-336.

Ishii, T., Bannai, S. & Sugita, Y. (1981) Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol *in vitro*. J. Biol. Chem. 23: 12387-12392.

Iwata, S., Hori, T., Sato, N., Udea-Taniguchi, Y., Yamabe, T., Nakamura, H., Masutani, H. & Yodoi, J. (1994) Thiol-mediated redox regulation of lymphocyte proliferation. J. Immunol. 152: 5633-5642.

Jaeschke, H. & Wendel, A. (1985) Diurnal fluctuation and pharmacological alteration of mouse organ glutathione content. Biochem. Pharmacol. 34:1029-1033.

Kavanagh, T.J., Grossmann, A., Jaecks, E.P., Jinneman, J.C., Eaton, D.L., Martin, G.M. & Rabinovitch, P.S. (1990) Proliferative capacity of human peripheral blood lymphocytes sorted on the basis of glutatione content. J. Cell Physiol. 145: 472-480.

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

Khalil, M., Kabiel, A., El-Khateeb, S., Aref, K., El Lozy, M., Jahnin, S. & Nasr, F. (1974) Plasma and red cell water and elements in protein-calorie malnutrition. Am. J. Clin. Nutr. 27: 260-267.

King, L.E. & Fraker, P.J. (1991) Flow cytometric analysis of the phenotypic distribution of splenic lymphocytes in zinc-deficient adult mice. J. Nutr. 121: 260-267.

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

Kramer, T.R. (1984) Reevaluation of zinc deficiency on Concanavalin-A-induced rat spleen lymphocyte proliferation. J. Nutr. 114: 953-963.

Lee, W.H. & Woodward, B.D. (1996) The CD4/CD8 ratio in the blood does not reflect the response of this index in secondary lymphoid organs of weanling mice in models of protein-energy malnutrition known to depress thymus-dependent immunity. J. Nutr. 126: 849-859.

Lepage, L.M. (1997) Effects of dietary zinc deficiency and malnutrition on the T-lymphocyte zinc-finger protein $p56^{lck}$ in mice. MSc. Thesis. Department of Foods and Nutrition, University of Manitoba, Winnipeg, Manitoba.

Luecke, R.W., Simonel, C.E. & Fraker, P.J. (1978) The effect of restricted dietary intake on the antibody mediated response of the zinc deficient A/J mouse. J. Nutr. 108: 881-887.

Mares-Perlman, J.A., Subar, A.F., Block, G., Greger, J.L. & Luby, M.H. (1995) Zinc intake and sources in the US adult population: 1976-1980. J. Amer. Col. Nutr. 14: 349-357.

Maret, W. (1994) Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. Proc. Natl. Acad. Sci. USA. 91: 237-241.

Mayatepek, E., Becker, K., Gana, L., Hoffmann, G. & Leichsenring, M. (1993) Leukotrienes in the pathophysiology of kwashiorkor. Lancet. 342: 958-960.

Mengheri, E., Viglolini, F. & Gaetani, S. (1995) Zinc deficiency affects the expression of IL-2 but not of IL-2R in spleen lymphocytes of rats. Nutr. Rev. 15: 505-515.

Messina, J.P. & Lawrence, D.A. (1989) Cell cycle progression of glutathione-depleted human peripheral blood mononuclear cells is inhibited at S phase. J. Immunol. 143: 1974-1981.

Meuer, S.C., Hussey, R.E., Cantrell, D.A., Hodgdon, J.C., Schlossman, S.F., Smith, K.A. & Reinherz, E.L. (1984) Triggering of the T3-T1 antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway. Proc. Natl. Acad. Sci. USA. 81: 1509-1513.

Miller, L.V., Hambridge, K.M., Naake, V.L., Hong, Z., Westcott, J.L. & Fennessey, P.V. (1994) Size of the zinc pools that exchange rapidly with plasma zinc in humans: Alternative techniques for measuring and relation to dietary zinc intake. J. Nutr. 124: 268-276.

Mills, B.J., Lindeman, R.D. & Lang, C.A. (1981) Effect of zinc deficiency on blood glutathione levels. J. Nutr. 111: 1098-1102.

Moulder, K. & Steward, M.W. (1989) Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody. Clin. Exp. Immunol. 77: 259-274.

Petro, T.M. (1985) Effect of reduced dietary protein intake on regulation of murine in vitro polyclonal T lymphocyte mitogenesis. Nutr. Res. 5: 263-276.

Pocino, M., Malave, I. & Baute, L. (1992) Mitogenic effect of zinc on lymphocytes from strains of mice that are either high or low-responder to T-cell mitogens. Immunopharmac. Immunotoxicol. 14: 295-321.

Poot, M., Teubert, H., Rabinovitch, P.S. & Kavanagh, T.J. (1995) De novo synthesis of glutathione is required for both entry into and progression through the cell cycle. J. Cell. Physiol. 163: 555-560.

Prasad, A.S. (1996) Zinc deficiency in women, infants and children. J. Amer. Col. Nutr. 15: 113-120.

Prasad, A.S. (1995) Zinc: An overview. Nutrition. 11: 93-99.

Prasad, A.S., Miale, A., Farid, Z., Sandstead, H.H. & Schulert, A.R. (1963) Zn metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, and hypogonadism. J. Lab. Clin. Med. 61: 537-549.

Reddy, G.P.V. (1994) Cell cycle: Regulatory events in $G_1 \rightarrow S$ transition of mammalian cells. J. Cell. Biochem. 54: 379-386.

Rhodes, D. & Klug, A. (1993) Zinc fingers. Sci. Amer. 268: 56-65.

Salvin, S.B. & Rabin, B.S. (1984) Resistance and susceptibility to infection in inbred murine strains. Cell. Immunol. 87: 546-552.

Sandstead, H.H. (1995) Is zinc deficiency a public health problem? Nutrition. 11: 87-92.

Seagrave, J., Tobey, R.A. & Hildebrand, C.E. (1983) Zinc effects on glutathione metabolism relationship to zinc-induced protection from alkylating agents. Bichem. Pharmac. 32: 3017-3021.

Smythe, P.M., Schlonland, M., Brereton-Stiles, G.G., Coovadia, H.M., Grace, H.J., Loenig, W.E.K., Mafoyane, A., Parent, M.A. & Vos, G.H. (1971) Thymolymphatic

deficiency and depression of cell-mediated immunity in protein-calorie malnutrition. Lancet. Oct: 939-943.

Staal, F.J.T., Roederer, M., Herzenberger, L.A. & Herzenberger, L.A. (1992) Glutathione and immunophenotypes of T and B lymphocytes in HIV-infected individuals. Ann NY Acad Sci. 651: 453-463.

Tanaka, Y., Shiozwaw, S., Morimoto, I. & Fujita, T. (1990) Role of zinc in interleukin 2 (IL-2)-mediated T-cell activation. Scand. J. Immunol. 31: 547-552.

Taylor, C.G., Potter, A.J. & Rabinovitch, P.S. (1997) Splenocyte glutathione and CD3-mediated cell proliferation are reduced in mice fed a protein-deficient diet. J. Nutr. 127: 44-50.

Telford, W. G. & Fraker, P.J. (1995) Preferential induction of apoptosis in mouse CD4+ CD8+$\alpha\beta$TCR^{lo}CD3ε^{lo} thymocytes by zinc. J. Cell. Physiol. 164: 259-270.

Vallee, B.L. & Falchuk, K.H. (1993) The biochemical basis of zinc physiology. Physiol. Rev. 73: 79-118.

Woodward, B.D. & Miller, R.G. (1991) Depression of thymus-dependent immunity in wasting protein-energy malnutrition does not depend on an altered ratio of helper ($CD4^+$) to suppressor ($CD8^+$) T cells or on a disproportionately large atrophy of the T-cell relative to the B-cell pool. Am. J. Clin. Nutr. 53: 1329-1335.

Woodward, B.D., Woods, J.W. & Crouch, D.A. (1992) Direct evidence that primary acquired cell-mediated immunity is less resistant than is primary thymus-dependent humoral immunity to the depressive influence of wasting protein-energy malnutrition in weanling mice. Am. J. Clin. Nutr. 55: 1180-1185.

Zhou, J.R., Canar, M.M. & Erdman, J.W. (1993) Bone zinc is poorly released in young, growing rats fed marginally zinc-restricted diet. J. Nutr. 123:1383-1388.

Zmuda, J. & Friedenson, B. (1983) Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. J. Immunol. 130: 362-364.

Zwickl, C.M. & Fraker, P.J. (1980) Restoration of the antibody mediated response of zinc/caloric deficient neonatal mice. Immunol. Commun. 9: 611-626.

APPENDIX A

METHOD DEVELOPMENT

A method for murine lymphocyte cell cycle analysis using BrdU incorporation and flow cytometry

One of the objectives of this thesis was to determine if dietary deficiencies of zinc and protein, alone or in combination, interfere with the ability of murine splenocytes to progress through the cell cycle. The method chosen for analysis of cell cycle progression was the incorporation of bromodeoxyuridine (BrdU) into proliferating cells in culture. BrdU is an analog of thymidine that replaces thymidine in cycling cells. A flourescent-labeled anti-BrdU antibody is used to identify the BrdU that has been incorporated into DNA of cells that have undergone DNA synthesis in the presence of BrdU. Due to the fact that the anti-BrdU antibody only binds to single-stranded DNA, the DNA must be partially denatured prior to the labeling procedure. The proportion of cells in the S-phase of the cell cycle can then be determined by detecting the flourescent-labelled anti-BrdU antibody by flow cytometry. This method is usually used in combination with a propidium iodide (PI) stain, which stains total cellular DNA red, allowing for the distinction of cells in the G_0, S and G_2/M phases of the cell cycle.

The BrdU procedure was the method of choice for cell cycle analysis because it is a direct measure of cell proliferation and offers many advantages over other previously reported, less direct methods of cell proliferation or DNA synthesis (Dolbeare et al, 1983). One advantage is that no radioactivity is involved in the procedure. Another advantage is that the analysis time is very short, generally less than 5 minutes per sample.

Also, very few cells are required for analysis (only a few million cells per sample), which was very important in this case, as the number of lymphocytes available from a murine spleen is fairly low. Because it is easy to distinguish between BrdU-labeled and BrdU-unlabeled cells, there is little difficulty in identifying S-phase cells. Another definite advantage was the availability of the flow cytometer at the University of Manitoba.

Unfortunately, the BrdU incorporation method has been used primarily for cell culture lines. No studies were found where the procedure was used for murine splenocytes. Therefore, an optimized procedure for cell cycle analysis in murine splenocytes had to be developed. There are a number of different BrdU procedures in the literature, with the main variation among them being the type of denaturation procedure used. Denaturation has been performed by incubating the cells in a high-molarity (2M) HCl solution for 30 minutes at room temperature (Dolbeare et al, 1983), by incubating the cells in a low molarity (0.1M) HCl solution for 10 minutes followed by heating the cells in a boiling water bath for 10 minutes (Dolbeare et al, 1990), or by using restriction endonucleases in combination with exonuclease III (Dolbeare & Gray, 1988). Two other main variables to be determined were the time of incubation and the length of the BrdU pulse (ie. the length of time that BrdU was present in the culture). The following is a discussion of the events leading to the development of an optimized procedure for BrdU cell cycle analysis of murine spleen cells. The details of the reagents and equipment that were used for the optimized procedure are included in the Methods and Materials section.

Cells were originally cultured in 96-well microtiter plates (250 000-500 000 cells per well) and stimulated with 2 μg/ml anti-CD3 (clone 145 2C11, Cedarlane Laboratories

Ltd., Hornby, ON). Anti-CD3 was diluted in PBS, 100µl was plated into the wells and allowed to sit for 30 minutes at room temperature, after which time the fluid was aspirated off. The principle underlying this procedure is that the anti-CD3 adheres to the bottom of the plate, so when the fluid is aspirated off, the anti-CD3 remains in the wells. When cells are cultured, they settle to the bottom of the wells and form a matrix with the plate-bound anti-CD3. Stimulation of T-lymphocytes has been shown to occur using this method (Meuer et al, 1984). The incubation time used originally was 72 hours, with addition of BrdU to the media at the time the cells were cultured.

The BrdU labelling method first used was the protocol supplied with the anti-BrdU antibody (mouse monoclonal antibody to BrdU, clone BMC 9318, conjugated to flourescein solution) originally purchased from Boehringer Mannheim (Indianapolis, IN). This method involved fixing the harvested cells in 70% ethanol, followed by resuspending the cells in 1 ml of chilled 0.1M HCl containing 0.5% Triton X-100 and incubating the cells on ice for 10 minutes. Following this, the cells were diluted with distilled water, centrifuged at 200 x g for 10 minutes and resuspended in 2 ml distilled water. The cellular DNA was then denatured by submerging the cell suspension into a boiling water bath for 10 minutes. Afterwards, the cells were quickly cooled by placing the tubes on ice for several minutes. The cells were then washed in PBS containing 0.5% Triton X-100, incubated with the anti-BrdU antibody for 30 minutes, centrifuged for one minute at 14000 RPM. The final step was staining with 300 µl of PI/PBS solution (5µg/ml).

The problems encountered after this first attempt were numerous. The protocol was very vague as to the specifics of the cell harvesting technique, and reviews of the

literature did not reveal much more information. Therefore, the cells were simply harvested by drawing the media through a pipet tip numerous times in an attempt to dislodge the cells, followed by removal of the media directly into the sample tubes. However, when the samples were analyzed under a microscope at the end of the BrdU-labelling procedure, very few cells were present. This may have been a combination of poor cell harvesting and the fact that plastic cell culture tubes were used for the entire procedure, which may have contributed to cell loss. Another problem encountered was that the cells that were present, when viewed under a microscope, were found to be excessively clumped. The clumping observed was most likely due to the fact that the cells were added to the 70% ethanol solution at one time, without vortexing. The purpose of the ethanol solution is to fix the cells and permeabilize the membrane so that the anti-BrdU can penetrate the cells and bind to the DNA inside the cell. If cells are added all at once, the cells will be fixed in a clump. It is very important that cells are added slowly to the ethanol solution and then quickly dispersed by vortexing, so that they are fixed as single cells. Also, the ethanol should be prepared ahead of time and stored at -20°C until use (Rector, E June 17, 1996, personal communication). Dr. Rector also recommended the protocol provided by Becton Dickinson which uses a more concentrated HCl solution in the DNA denaturation step, but involves no heat treatment.

For the next attempt, using the Becton Dickinson method, cells were cultured and harvested using the method described above. Following harvest, the cells were washed twice in PBS/1%BSA at 500 x g for 15 minutes at room temperature in plastic culture tubes. The cells were then resuspended in 100 µl of normal saline on ice and added dropwise to glass tubes containing 5 ml cold 70% ethanol while maintaining a vortex.

The tubes were then incubated on ice for 30 minutes, followed by centrifugation at 500 x g for 10 minutes at 10°C. The supernatant was aspirated and the tube was vortexed while 1 ml of 2 N HCl/0.5% Triton X-100 was added to the cells. Addition of HCl to a cell pellet will cause the cells to aggregate, so vortexing is necessary to prevent this (Becton Dickinson protocol). The cells were then incubated at room temperature for an additional 30 minutes, followed by centrifugation at 500 x g for 10 minutes at room temperature. The cells were then resuspended in 1 ml 0.1 M $Na_2B_4O_7 \cdot 10\ H_2O$, pH 8.5, to neutralize the acid. At this point, the cells were resuspended in 70% ethanol and stored at -20°C overnight. The Becton Dickinson protocol indicates that this method may be used to store cells. The next day, the cells were centrifuged, the pellet was resuspended in 50 µl of PBS/1% BSA/0.5% Tween 20 and transferred to 1.5 ml microcentrifuge tubes. At this point, 5 µl of anti-BrdU was added to each sample and the samples were incubated at room temperature for 30 minutes. This was followed by centrifugation at 1400 RPM for 1 minute and resuspension in 5 µg/ml of PI for flow cytometric analysis. Unfortunately, microscopic analysis revealed that there were very few cells in each sample, and that the cells that were present were clumped together.

The next step was an attempt to increase cell numbers at the end of the procedure, simply by starting off with more cells in each sample. Each well contained 500 000 cells and six wells were combined, providing $3x10^6$ cells per sample. This attempt was intended to compare two different incubation times (30 minutes and 72 hours), as well as to give some indication as to the length of time that the cells should be exposed to BrdU (30 minutes and 72 hours). The cells were cultured, harvested and processed according to the methods used in the latest attempt, with increased vortexing at the HCl addition

stage. The cells were stored in ethanol overnight, prior to staining with anti-BrdU. Once again, microscopic analysis revealed very few cells and lots of clumping. Unfortunately, no information regarding optimal incubation times or BrdU pulse lengths could be obtained until the problem of cell numbers and cell clumping was resolved.

To address whether the cells were being lost through the various centrifugation steps in the BrdU staining procedure, or whether it was a problem with the cell harvesting method, fresh spleen cells were isolated and processed according to the Becton Dickinson protocol for labeling cells with BrdU, without being put into culture. Microscopic analysis revealed that high numbers of cells remained throughout the entire procedure, indicating that cells were not being effectively harvested from the bottom of the wells of the microtiter plate. It was obvious at this point that the cell harvesting method must be addressed before continuing with the procedure.

It was suggested that the media be removed from the wells, and replaced with 100 µl of cold PBS. The plate was then allowed to sit at room temperature for 5-10 minutes, the bottom of the wells gently scraped with a pipet, followed by the removal of the PBS into a sample tube (Nayak, personal communication). The cells were again processed according to the Becton Dickinson protocol, and stored in ethanol overnight prior to staining with anti-BrdU. When the cells were viewed under a flourescent microscope, there appeared to be a slight increase in cell number and lots of PI staining, however, the cells did not appear to be intact.

At this point it was decided to switch from using round-bottomed plastic cell culture tubes for the initial steps and round-bottomed glass tubes for the steps following ethanol fixation, to using plastic conical Falcon centrifuge tubes for the entire procedure.

It was thought that the cell pellet may not be as easily lost when discarding supernatant fractions from a conical tube as from a round-bottomed tube. Also, the cells adhere slightly to the plastic of the Falcon tubes. This improved cell numbers quite a bit, so some cell losses must have been occurring due to the type of tubes being used. However, although cell number seemed to have increased, the cells did not look healthy under a microscope. There appeared to be a lot of dead cells and debris. Some cells were harvested after 72 hours of culture and observed with Trypan Blue, which revealed that the majority of the cells were dead when they were removed from culture. This brought into question the length of culture required for the stimulation of lymphocytes. It was decided to try reducing the length of culture to as low as 12 hours.

At this point it was decided to try using a commercially available non-enzymic solution, called cell dissociation solution (Sigma Chemical Co., St. Louis, MO), to increase the number of cells removed from the bottom of the microtiter plate during cell harvesting. The procedure remained essentially the same as described above using PBS, except this time 100 µl of cell dissociation solution was added to each well. This seemed to increase cell numbers slightly, but still was not satisfactory. It was then decided to switch from the 96-well plates to 24-well plates so that the wells could be scraped with a plastic cell scraper while incubating with cell dissociation solution to physically remove the cells from the bottom of the well. Also, this would eliminate the need to pool wells in order to obtain $3x10^6$ cells per sample, as $3x10^6$ cells could be plated in each well of the 24-well plate. This seemed to improve cell numbers drastically, as enough cells were present to be able to run the samples through the flow cytometer (up until this point, samples were usually just viewed under a flourescent microscope, but not run through

flow cytometer due to low cell numbers and problems with cell clumping). Unfortunately, the cells still seemed to be fragmented and broken, and very few intact cells were detected by the flow cytometer.

In consultation with another graduate student doing similar work with muscle cells, it was decided to eliminate the Tween 20 from the PBS/BSA solution. The cells were harvested using the cell dissociation solution and the cell scraper, and then processed according the Becton Dickinson protocol, with the elimination of Tween 20 from all solutions. The results were excellent, as the numbers of intact cells increased dramatically and very little clumping was observed. The concentration of Tween 20 may have been too harsh of a detergent for the lymphocytes.

Now that it was finally possible to run enough intact cells through the flow cytometer, attention focused on determining the appropriate duration of incubation and the length of the BrdU pulse. As well, other problems had arisen. There did not seem to be any sign of stimulation of the cells with anti-CD3 (according to the PI staining pattern), and there also did not appear to be any sign of staining with the FITC-conjugated anti-BrdU. A new anti-BrdU antibody was ordered from Boehringer Mannheim, but still no staining was detected. A different anti-BrdU antibody was ordered from Becton Dickinson and staining was observed. It was concluded that the Boehringer Mannheim product was defective.

Now that the BrdU labeling procedure was essentially working, it was very obvious that the cells were not being stimulated by the anti-CD3 plate-coating procedure. Numerous incubation periods were tested, including 24, 32, 48 and 72 hours of culture with BrdU in the media for the entire culture time (to ensure that the reason that no BrdU

staining was being detected was not due to a suboptimal exposure period of BrdU to the cells in culture). The cells were harvested and processed according to the modified procedure as described thus far, and stored in ethanol overnight prior to analysis by flow cytometry. Cells were not stimulated even after 72 hours of culture. It was decided to try stimulating the T-lymphocytes with varying concentrations of anti-CD3 (0.5, 2, 5 & 10 μg/ml), and also to try using two different lectins, Concanavalin A(ConA) and phytohemagglutinin (PHA), at three different concentrations (2.5, 5 & 10 μg/ml) for 24 hours. No activity was seen at any of the anti-CD3 concentrations, while stimulation was observed in samples stimulated with either 5 or 10 μg/ml of ConA or PHA. It was decided to compare the degree of stimulation of T-lymphocytes with 5 and 10 μg/ml of both ConA and PHA with culture times of 24, 48 & 72 hours. Also, it was decided to process and analyze cells in one day, without storage overnight in ethanol, to see if this would result in a sample with less cellular debris and more intact cells.

Analysis revealed that stimulation with 5 μg/ml ConA for 48 hours resulted in the most stimulation (See Appendix A, Table 1), while maintaining relatively high numbers of intact cells with a limited amount of debris. Increasing culture time to 72 hours resulted in increased cell destruction. It was also decided that samples should be analyzed on the same day that they are removed from culture, to eliminate cell destruction that occurs during storage in ethanol. However, it was revealed that BrdU should not be present in the media for the entire duration of culture, as strange results were seen. After experimenting with a variety of BrdU pulse times, it was decided that a four hour pulse was the most effective (that is BrdU is added to the media 4 hours before the cells are removed from culture).

The amount of BrdU added to the media was also increased to twice the original amount after experimentation with 1x, 2x, 4x & 10x the original amount (20µl/well). See Appendix A, Table 1.

In summary, the optimized procedure is as follows:

- Culture $3x10^6$ cells/well in a 24-well culture plate
- Stimulate cells with 5 µg/ml ConA added at beginning of culture period
- 4 hours prior to removing from culture, add 40 µl BrdU/well
- After 48 hours culture, replace media with 1 ml cell dissociation solution and scrape wells with cell scraper
- Pipet samples into 15 ml conical plastic Falcon tubes and and add 1 ml PBS/1% BSA
- Centrifuge at 500 x g for 15 minutes
- Resuspend pellet in 100 µl physiological saline on ice
- Add cells dropwise to cold 70% ethanol in conical Falcon tube, while vortexing
- Incubate for 30 minutes on ice
- Centrifuge at 500 x g for 10 minutes at 10°C
- Resuspend pellet in 500 µl 2N HCl/0.5%Triton X-100 while vortexing
- Incubate for 30 minutes at room temperature
- Centrifuge at 500 x g for 10 minutes at room temperature
- Resuspend pellet in 500 µl $Na_2B_4O_7 \cdot 10\ H_2O$

- Centrifuge at 500 x g for 10 minutes at room temperature
- Resuspend the pellet in 50 µl PBS/1% BSA and transfer to 1.5 ml microcentrifuge tubes
- Add 20 µl anti-BrdU antibody
- Incubate for 30 minutes at room temperature
- Centrifuge at 14 000 RPM for 1 minute
- Resuspend pellet in 300 µl PI solution (5 µg/ml PBS)
- Analyze by flow cytometry within 3 hours

Total percentage of cells in S phase after 48 hours of culture

ConA concentration		
2.5 *u* g/ml	5 *u* g/ml	10 *u* g/ml
3.22	16.88	46.76[1]

	BrdU concentration[2]			
	10 mM	20 mM	40 mM	100 mM
4 hour BrdU pulse	8.66	22.44	15.00	16.12
2 hour BrdU pulse	14.02	13.55	-	-

[1]Abnormal histogram pattern for this sample.

[2]Cells were stimulated with 5 *u* g/ml ConA.

Appendix B

Average weekly weights of mice for Experiments 1 & 2

	Treatment Groups[2]									
	Experiment 1					Experiment 2				
	ZnDF&LP	ZnDF	LP	ER	CTRL	ZnDF&LP	ZnDF	LP	ER	CTRL
Initial Weight	23.4 ±0.49	22.8 ±0.39	23.4 ±0.61	23.6 ±0.49	23.6 ±0.40	23.3 ±0.37	23.1 ±0.22	22.8 ±0.24	23.4 ±0.36	23.6 ±0.47
Acclim Weight[3]	ND[4]	ND	ND	ND	ND	21.8^{A} ±0.73	$20.3^{A,B}$ ±0.70	$20.0^{A,B}$ ±1.05	20.6^{B} ±0.60	$21.7^{A,B}$ ±0.56
Week 1	19.2 ±0.49	19.9 ±0.90	19.4 ±0.89	21.3 ±0.71	20.9 ±0.51	$21.0^{A,B}$ ±0.72	$21.9^{A,B}$ ±0.48	$20.1^{A,B}$ ±0.70	19.6^{B} ±0.75	22.4^{A} ±0.92
Week 2	18.1^{C} ±0.79	$20.2^{A,B,C}$ ±0.73	$19.3^{B,C}$ ±0.78	$21.1^{A,B}$ ±0.71	21.6^{A} ±0.57	$19.5^{B,C}$ ±0.54	22.8^{A} ±0.31	17.7^{C} ±0.79	20.3^{B} ±0.55	23.9^{A} ±1.00
Week 3	18.1^{C} ±1.05	$21.0^{A,B}$ ±0.57	$19.2^{B,C}$ ±0.89	$21.4^{A,B}$ ±1.04	22.8^{A} ±0.38	19.4^{C} ±0.72	$22.3^{A,B}$ ±0.58	18.3^{C} ±0.86	$20.5^{B,C}$ ±0.40	25.1^{A} ±1.17
Week 4	17.4^{C} ±1.19	21.0^{B} ±0.62	$19.5^{B,C}$ ±1.03	20.1^{B} ±0.85	23.5^{A} ±0.35	19.4^{C} ±0.67	21.7^{B} ±0.72	18.6^{C} ±0.81	$21.0^{B,C}$ ±0.36	25.5^{A} ±1.00

[1]Values are expressed as mean ± the standard error of the mean. For Experiment 1, n=7, except for ZnDF&LP , where n=5. For Experiment 2, n=8, except for LP, where n=6. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. Superscript letters indicate significant differences between means for each experiment as determined by Duncan's multiple range test.

[2]ZnDF&LP=Zn deficient and 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[3]Acclim weight=weight after acclimatization for one week in hanging cages fed powdered control diet.

[4]ND=not determined as part of the experimental design for Experiment 1.

Appendix C

Effects of dietary treatments on femur, serum and spleen zinc concentration in mice after 4 weeks[1]

	Treatment Groups[2]				
	ZnDF&LP	ZnDF	LP	ER	CTRL
Serum Zn (Exp 1)[3] (*u*g/ml)	0.52^{B} ±0.06	0.56^{B} ±0.09	1.01^{A} ±0.03	1.15^{A} ±0.13	1.14^{A} ±0.11
Serum Zn (Exp 2)[4] (*u*g/ml)	0.75^{C} ±0.09	0.75^{C} ±0.14	$0.90^{B.C}$ ±0.13	$1.22^{A.B}$ ±0.07	1.36^{A} ±0.11
Femur Zn (Exp 2)[5] (*u*g/g dry wt)	264.1 ±20.9	254.8 ±18.4	268.2 ±25.0	249.4 ±12.4	306.2 ±28.7
Spleen Zn[6] (*u*g/g dry wt)	415.5 ±158.8	761.1 ±271.9	502.8 ±164.2	431.4 ±105.1	366.1 ±44.9

[1]Values are means ± standard error of the mean. Main effects of diet were significant for serum Zn in Experiment 1 and 2. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[3]Values are from mice in Experiment 1. n=3, except for ZnDF&LP, and CTRL, where n=2 and 4, respectively. Values represent pooled samples.

[4]Values are from mice in Experiment 2. n=7, except for ZnDF&LP, ZnDF and LP, where n=6, 8 and 5, respectively.

[5]Values are from mice in Experiment 2. n=7, except for LP and ER, where n=5 and 8, respectively.

[6]Values are from a separate group of mice fed according to the same protocol as for Experiment 1. n=5 for all groups.

Appendix D

Effects of dietary treatments on spleen weight and spleen cell numbers in mice from Experiment 1 after 4 weeks[1]

	Treatment group[2]					
	B	ZnDF&LP	ZnDF	LP	ER	CTRL
Spleen weight (mg)	104.0^{A} ±7.90	56.5^{B} ±6.20	74.9^{B} ±4.70	70.7^{B} ±6.60	73.3^{B} ±7.80	114.5^{A} ±9.20
% Spleen/Body weight	0.45^{A} ±0.03	0.31^{B} ±0.02	0.35^{B} ±0.02	0.36^{B} ±0.02	0.36^{B} ±0.03	0.49^{A} ±0.03
Splenocytes/ spleen ($x10^{8}$)	$1.56^{A,B}$ ±0.27	0.98^{B} ±0.26	$1.33^{A,B}$ ±0.15	$1.28^{A,B}$ ±0.29	$1.38^{A,B}$ ±0.23	2.15^{A} ±0.49
Splenocytes/ mg spleen ($x10^{6}$)	1.50 ±0.23	1.60 ±0.29	1.79 ±0.20	1.71 ±0.26	1.59 ±0.30	1.83 ±0.32

[1]Values are means ± the standard error of the mean. n=7, except for ZnDF&LP, where n=5. Main effect of diet was significant for spleen weight and spleen weight as a percent of body weight. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]B=baseline, ZnDF&LP=Zn deficient and 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

Appendix E

Effects of dietary treatments on T-lymphocyte subpopulations[1]

	Treatment Groups[2]					
	B	ZnDF&LP	ZnDF	LP	ER	CTRL
% CD3+[3]	13.8^{B} ±2.5	18.1^{A,B} ±4.3	16.9^{A,B} ±6.3	19.6^{A} ±6.1	20.1^{A} ±5.0	14.6^{B} ±5.2
% CD4+[4]	57.2^{A} ±1.3	42.9^{C} ±5.1	49.8^{B} ±2.3	51.7^{A,B} ±1.7	51.1^{A,B} ±2.9	55.9^{A,B} ±2.2
% CD8+[4]	15.51 ±0.9	16.3 ±2.1	14.6 ±1.5	14.7 ±1.1	14.7 ±1.1	16.8 ±1.3
Total % CD4+ and CD8+[4]	72.7^{A} ±1.5	59.2^{C} ±5.2	64.4^{B,C} ±2.1	66.4^{B} ±1.6	65.8^{B} ±3.4	72.7^{A} ±1.9
CD4+/CD8+ ratio	3.76 ±0.2	2.8 ±0.5	3.7 ±0.6	3.7 ±0.4	3.57 ±0.3	3.5 ±0.4
%CD25+[5]	1.45 ±0.07	1.35 ±0.22	1.40 ±0.13	1.47 ±0.12	1.69 ±0.21	1.47 ±0.14

[1]Values are means ± standard error of the mean. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test. There was a significant main effect of diet for CD4+, and total CD4+ and CD8+, and a significant main effect of day for CD3+, CD4+, CD8+, and total CD4+ and CD8+. n=7, except for ZnDF&LP, where n=5.

[2]B=baseline, ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[3] Expressed as % of total splenocytes.

[4] Expressed as % of CD3+ splenocytes.

[5]Expressed as % of total lymphocytes.

Appendix F

Effects of dietary treatments on spleen weight and spleen cell numbers in mice from Experiment 2 after 4 weeks[1]

	Treatment group[2]				
	ZnDF&LP	ZnDF	LP	ER	CTRL
Spleen weight[3] (mg)	78.1^{B} ±6.74	87.8^{B} ±6.83	70.3^{B} ±10.09	73.6^{B} ±4.98	112.0^{A} ±5.20
% Spleen/Body weight[3]	$0.40^{A,B}$ ±0.02	$0.40^{A,B}$ ±0.02	$0.37^{A,B}$ ±0.04	0.35^{B} ±0.02	0.44^{A} ±0.02
Splenocytes/ spleen[4] ($x10^{8}$)	$1.22^{A,B}$ ±0.31	$1.26^{A,B}$ ±0.22	1.03^{B} ±0.15	$1.24^{A,B}$ ±0.49	2.09^{A} ±0.33
Splenocytes/ mg spleen[4] ($x10^{6}$)	1.63 ±0.05	1.35 ±0.02	1.35 ±0.02	1.52 ±0.06	1.87 ±0.03

[1]Values are means ± the standard error of the mean. Main effects of diet were significant for spleen weight. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]ZnDF&LP=Zn deficient and 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[3]n=8, except for LP, where n=6.

[4]n=7, except for LP, ER and CTRL, where n=5, 4 and 8, respectively.

Appendix G

Effects of dietary treatment and 2-mercaptoethanol treatment in vitro on the percentage of ConA-stimulated murine spleen cells in each phase of the cell cycle[1,2]

Treatment Group[3]	Culture Conditions									
	+2-ME					-2-ME				
	G_0	Early S	Late S	Total S	Mitosis	G_0	Early S	Late S	Total S	Mitosis
ZnDF&LP[4]	78.75^{A} ±3.31	9.51^{B} ±1.80	7.69^{B} ±1.39	17.21^{B} ±2.91	4.06 ±0.59	86.72 ±2.58	4.70 ±0.84	3.62 ±1.51	8.34 ±2.22	4.95^{B,C} ±0.63
ZnDF	79.79^{A} ±2.49	8.44^{B} ±1.27	7.38^{B} ±1.58	15.82^{B} ±2.48	4.44 ±0.52	87.83 ±1.59	4.73 ±1.09	2.66 ±0.54	7.38 ±1.47	4.80^{C} ±0.45
LP	80.03^{A} ±1.30	8.46^{B} ±1.97	7.32^{B} ±0.91	15.77^{B} ±1.70	4.13 ±0.58	86.95 ±1.26	3.81 ±0.78	2.68 ±0.66	6.50 ±1.42	6.56^{A} ±0.79
ER	76.36^{A,B} ±4.49	9.85^{A,B} ±2.17	9.07^{A,B} ±2.74	18.92^{B} ±4.24	4.72 ±0.77	84.16 ±0.18	5.14 ±0.36	4.34 ±0.83	9.48 ±0.96	6.37^{A,B} ±0.85
CTRL	70.36^{B} ±1.85	13.55^{A} ±1.84	12.18^{A} ±1.53	25.72^{A} ±1.86	3.62 ±0.66	86.66 ±1.11	4.42 ±0.67	3.91 ±0.51	8.33 ±1.11	5.01^{B,C} ±0.37

[1]Values are means ± standard error of the mean. Different superscript letters indicate significant differences between means for a variable within a culture condition, as determined by Duncan's multiple range test.

[2]Significant main effects were stimulation and culture condition for all phases, except for M phase, where culture condition was not significant. When ANOVA was performed on stimulated samples only, a significant effect of day was found for G_0, early S, late S, and M phases for +2-ME samples and for G_0 and late S phases for -2-ME samples. A significant effect of diet was found for G_0, early S, late S, and total S phases for +2-ME samples. No significant diet effects were found for cells cultured without 2-ME.

[3]ZnDF&LP=Zn deficient & 2% protein, ZnDF=Zn deficient, LP=2% protein, ER=energy restriction, CTRL=control.

[4]For +2-ME, n=6, 7, 5, 4 & 8 and for -2-ME, n=4, 6, 4, 3 & 7, for ZnDF&LP, ZnDF, LP, ER and CTRL, respectively.

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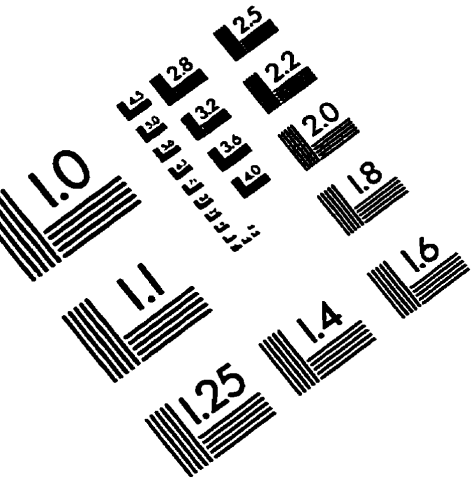

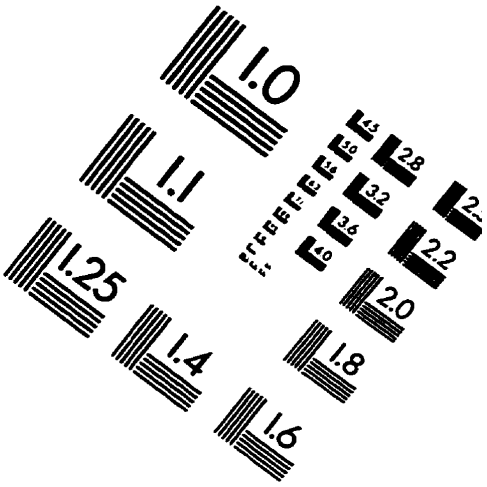

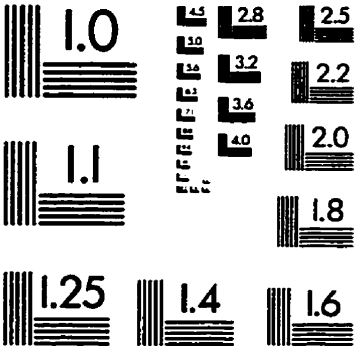

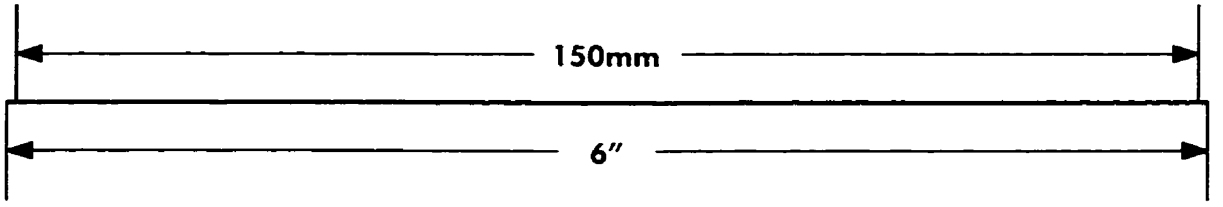

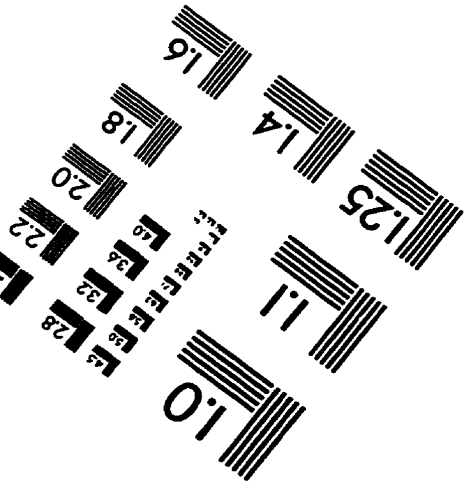

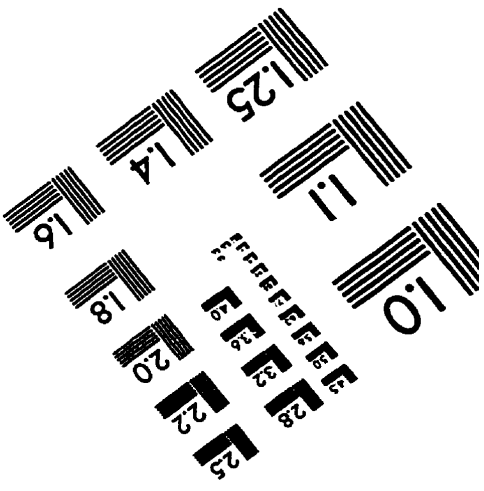

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