EFFECTS OF DIETARY ZINC DEFICIENCY AND PROTEIN-ENERGY MALNUTRITION ON MURINE SPLENIC T LYMPHOCYTE SIGNAL TRANSDUCTION PROTEINS

By

Jeri-Anne C. Giesbrecht

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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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

Effects of Dietary Zinc Deficiency and Protein-Energy Malnutrition on Murine Splenic T Lymphocyte Signal Transduction Proteins

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Malnutrition causes numerous detrimental alterations at the whole body, organ and cellular levels. Although the causative mechanisms are unknown, immune dysfunction often accompanies and compounds the effects of zinc deficiency, protein and energy malnutrition syndromes. T lymphocyte mediated immunity is particularly affected by zinc deficiency and is evidenced by reduced T lymphocyte numbers and reduced T lymphocyte proliferation. One mechanism by which zinc may exert this effect is through zinc metalloenzymes in the signal transduction pathway of T lymphocytes. In vitro studies indicate that zinc dependent proteins are detrimentally affected by chelation and point mutations. Phospholipase C (PLC) and protein kinase C (PKC) are zinc metalloenzymes in the T lymphocyte signal transduction pathway. It is hypothesized that aberrations in the expression and functionality of PLC and PKC due to a lack of zinc may help explain some of the immune dysfunction observed in zinc deficiency.

The objectives of the present studies were to assess the effects of zinc deficiency and malnutrition (energy, protein, or combined zinc and low protein) on animal weight, zinc status, spleen parameters, and the expression and activity of splenic T lymphocyte signal transduction proteins: PLC and PKC. An established adult murine model of zinc deficiency and protein and energy malnutrition was used to carry out these investigations.

In two separate experiments, 35 female C56BL/6 adult mice were randomly assigned to one of five treatment groups [zinc deficient and 2% protein (ZLP), 2% protein

(LP), zinc deficient (Z), energy restricted (ER), or control (CTL)] for four weeks. Zinc deficiency was induced in the Z and ZLP groups as assessed by lower serum and femur zinc concentrations compared to CTL (23%-65% and 78%-84% of CTL, respectively). LP mice also had a lower serum zinc concentration compared to CTL (0.52 ± 0.07 μg/ml versus 1.00 ± 0.07 μg/ml, Exp. 1). In both experiments, all deficient groups weighed significantly less than CTL (81%-90% of CTL), except for the Z group which was not significantly different from CTL. Similarly, spleen weight was significantly lower than CTL (69%-77% of CTL) in all groups except the Z group. There was a positive correlation ($r = 0.81$, $p = 0.0001$, Exp.1) between body weight and spleen weight indicating that spleen weight may respond to body weight status as opposed to pure nutrient deficiency. For Western Immunoblotting and activity assays, murine splenic T lymphocytes were isolated by use of immunocolumns. The expression of PLCγ1 and PKCα as well as the activity of PLC and PKC were not significantly different among treatment groups. Thus, it appears that zinc deficiency and protein and energy malnutrition syndromes do not have an effect on these T lymphocyte signal transduction proteins.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

I. Literature Review

Introduction

Zinc, as a nutrient, is known to have numerous functional roles and as such plays a part in most physiological systems, including the immune system. Although zinc deficiency is detrimental to the immune system, and in particular cell mediated immunity, the mechanisms by which zinc exerts this effect are largely unknown. One of the difficulties in addressing zinc deficiency is that it often occurs concomitant with protein-energy malnutrition. This literature review will discuss zinc and protein-energy malnutrition in relation to cell mediated immunity, specifically T lymphocyte signal transduction.

Nutrition and Immune Function

Since the early 1900s, when nutritional deficiencies were first discovered and the field of nutritional sciences began, it has been recognized that an adequate supply of nutrients is essential for any organism or biological system. The plethora of metabolic changes which accompanies malnutrition highlights the importance of nutrition for the organism or individual. Both macronutrient and micronutrient deficiencies can be devastating to the health and well-being of individuals and to societies in which these deficiencies are common and widespread.

Nutritional deficiencies are of concern as malnutrition is implicated in immune dysfunction and is the most common cause of immunodeficiency world wide (Gottschlich and Groziak, 1993). A chronic lack of nutrients sets the stage for the vicious cycle of malnutrition in which nutritional deficiencies make the host susceptible to infection

which then further compromises the nutritional status and health of the individual. Persons in this immunocompromised state are at a further disadvantage from the stand point of recovery if adequate nutrients can not be provided to the individual.

The immune system can be divided into two broad categories: (1) innate or nonspecific immunity which consists of lines of defense an individual is born with such as the physiological barrier of skin, and (2) acquired immunity which is specialized in order to mount a response against foreign particles (Roitt et al., 1998). Acquired immunity is further divided into either humoral or cell mediated immunity depending on the type of immune cells involved in facilitating the immune response. Humoral immunity is mediated by antibodies produced by B lymphocytes which mature in and are released from the bone marrow, whereas cell mediated immunity involves T lymphocytes that arise from stem cells in the bone marrow which migrate and mature in the thymus (Roitt et al., 1998). Humoral and cell mediated immunity act in concert to recognize foreign antigens and coordinate an immune response. Upon recognition of an antigen, T lymphocytes become activated which initiates an intracellular cascade of events leading to a cellular immune response. This intracellular cascade is known as a signal transduction pathway.

The specialized network of cells in the immune system have a high rate of turnover and rely on nutrients as cofactors in biochemical reactions (Chandra, 1991). Any condition that would limit the required nutrients would thereby reduce the ability of the immune cell to mount an immune response. In this way the immune system is highly susceptible to malnutrition resulting from macronutrient or micronutrient deficiencies.

The following sections will discuss zinc and the role of zinc deficiency in immune dysfunction, and in particular the aberrations in cell mediated immunity.

Zinc & Zinc Deficiency

i) Functional roles of zinc

Zinc is an essential trace element known to be involved in numerous physiological processes including growth and development, sexual maturation, membrane integrity and immunity (Prasad, 1995). The ability of zinc to affect such a broad spectrum of physiological activities is due to its numerous functions, particularly its role as an integral component of many proteins (Vallee and Falchuk, 1993). As a Lewis acid, zinc has strong binding affinity to electron donors such as amines and thiolates (Williams, 1989). As a result, zinc ions readily complex with amino acids, peptides, proteins, and nucleotides in a tetrahedral fashion such that four ligands are coordinated around the ion (King and Keen, 1999).

Over the last 30 years, the importance of zinc in metalloenzymes has been recognized in all six enzyme classes, and presently over 300 enzymes are known to require zinc. Within metalloenzymes zinc may have a catalytic, structural, or regulatory role. Catalytic involvement requires that the zinc ion is directly involved in enzyme catalysis such that enzyme activity is directly related to its metal content (Vallee and Falchuk, 1993). In addition, zinc is known to exhibit fast ligand exchange and thus is able to facilitate the catalytic process (Williams, 1989).

Alternatively, when zinc is involved in stabilizing the structure of a metalloenzyme, zinc is classified as having a structural role. More than ten classes of zinc based domains, with characteristic coordination and configuration of zinc with other

molecules, have been discovered (Berg and Shi, 1996). One of these zinc domains which has received much attention is the "zinc finger" in which a zinc atom is coordinated to two cysteine and two histidine (Cys_2,His_2) in such a fashion so that the protein strand is looped around zinc in what looks like a finger. Such a domain was first revealed in transcription factor IIIA, and subsequently in other proteins involved in gene regulation and otherwise (Berg and Shi, 1996). Zinc fingers and other domains are important in maintaining the three dimensional configuration of the protein and in facilitating interactions with other macromolecules.

Considering that zinc is found in more than 300 metalloenzymes, only a few studies have examined metalloenzyme function and zinc deficiency. While several zinc containing enzymes do not appear to be directly affected by zinc deficiency, a few enzymes have shown reduced activity. Specifically, the activity of alakaline phosphatase, a zinc metalloenzyme, has been shown to be significantly reduced in tissues including bone and thymus from zinc deficient rats when compared to their pair fed controls (Prasad and Oberleas, 1971). Another zinc dependent enzyme, thymidine kinase, which has a key role in DNA synthesis and cell division, has been shown to be reduced in zinc deficient animals compared to pair fed controls (Prasad and Oberleas, 1974). Thus, enzyme systems which are zinc dependent and tissues with high turnover rates may be sensitive to zinc deprivation.

ii) Zinc homeostasis and stores

Although the mechanism of absorption of zinc into enterocytes is largely unknown, it is presumed to cross the brush border via both carrier mediated and a non-carrier mediated mechanism (King and Keen, 1999). Zinc in the mucosal cell may then

be used for cellular processes, bound to metallothionein (MT) or pass through the cell via a carrier protein (Cousins, 1986). The primary homeostatic control for zinc is at the gut level where absorption or excretion can be modified such that retention is sufficient to meet the body's requirements (King and Keen, 1999). While this may help prevent toxicity of the mineral, deficiency states may be harder to defend against as there is still a high obligatory loss of endogenous zinc (Keen and Gershwin, 1990). Although the gut is able to provide some level of homeostatic control, continuous dietary intake is required as there are no specific body stores of zinc. It has been suggested that there is an exchangeable body pool of zinc and that zinc deficiency can cause a reduction of these pools (Miller et al., 1994).

Assessment of zinc status is difficult as there are few suitable diagnostic tests for humans. In animals, two commonly used indicators of zinc status are plasma or serum zinc, and femur zinc. Although serum zinc contains only 1% of the circulating total body zinc, it represents a source which is available to all tissues and which can be easily mobilized and taken up by tissues (Vallee and Falchuk, 1993). The plasma serves as the central compartment in which zinc is held and through which the exchangeable zinc from the exchangeable zinc pool can be passed (Miller et al., 1994). The rate of turnover in bone is much slower than in plasma or other tissues such as pancreas, liver, kidney and spleen (King and Keen, 1999). In addition, serum and femur are also important in immunity as stem cells are formed in the bone before they migrate to the thymus to become T lymphocytes which are then able to circulate throughout the body via the serum and lymph.

iii) Zinc deficiency in humans

While the essentiality of zinc in animals was recognized over 60 years ago, the importance of zinc in humans was not recognized until the 1960s when zinc deficiency was found in young Iranian males (Prasad et al., 1961; Prasad et al., 1963). These men presented with anemia, hypogonadism and growth retardation. Similar findings were also reported in adolescent boys from Egypt (Sandstead et al., 1967). Consumption of a cereal based diet and geophagia were considered to be the primary causes of zinc deficiency as diets with high phytate content are known to decrease the bioavailability of zinc. Since these early studies in the Middle East, zinc deficiency has been noted in several other areas including Guatemala, Chile, and disadvantaged socioeconomic groups of North America (Aggett and Comerford, 1995). In Denver, Colorado, Hambidge et al. (1972) found very low levels of zinc (<30 ppm) in hair to be associated with short stature, low weight, and poor appetite in infants and children. Similar findings were observed in Ontario when young boys with short stature were found to have a growth limiting zinc deficiency (associated with low hair zinc) and they experienced significantly improved linear growth ($p = 0.05$) with zinc treatment versus the placebo group (Gibson et al., 1989). Low dietary zinc intake was implicated as the cause of zinc deficiency in these Canadian children.

iv) Populations at risk for zinc deficiency

An individual or population group may be at risk for zinc deficiency for a number of reasons. Zinc may be lacking in the diet due to limited food selection and poor food choices. Components such as phytate and fiber act as competitive ligands for binding in gut and are known to inhibit zinc bioavailability while histidine, cysteine and digestible

proteins are known to enhance zinc absorption (Sandstead, 1991). Thus, populations which subsist on diets high in phytate and low in protein may be at risk of zinc deficiency. Disease may also induce zinc deficiency in certain patient populations. For example, zinc excretion is known to be increased in renal disease, alcoholism, sickle cell anemia, and states which produce muscle catabolism (Cunnane, 1988). Increased requirements are seen in malignancy, surgery and burn patients, and in times of growth such as childhood and pregnancy (Cunnane, 1988; King and Keen, 1999). Finally, malabsorption is also induced in persons with acrodermatitis enteropathica (AE), an inherited disease in which there is impaired intestinal uptake and transfer of zinc into the enterocyte (Keen and King, 1999). While normal adults have been shown to absorb 60-70% of a standard ^{65}Zn dose, children and adults with AE were only able to absorb 3% and 15-40%, respectively (in review by Vallee and Falchuk, 1993).

v) Signs of zinc deficiency

Many of the signs of zinc deficiency are not specific only to zinc but are common to many states of malnutrition. As with other deficiencies, the severity of signs corresponds to the severity of zinc deficiency. Characteristics of zinc deficiency, as were described in studies from the Middle East, include growth retardation, hypogonadism and delayed sexual maturation. Other manifestations include alopecia, skin and epithelial lesions, hypogeusia and anorexia, weight loss and tissue wasting, delayed wound healing and immune deficiencies (Keen and King, 1999). Alterations in immune function, specifically cell mediated immunity are significant and will be further examined in the section on Zinc and Immunity.

Protein Energy Malnutrition and Zinc Deficiency

A discussion of zinc deficiency would not be complete without reference to protein-energy malnutrition (PEM) as the two conditions may occur together and have similar clinical manifestations. "Protein-energy malnutrition results when the body's needs for protein, energy fuels, or both cannot be satisfied by the diet" (Keen and King, 1999). This broad definition has been used in the literature to describe several forms of malnutrition all under the common name of PEM. PEM can be further classified as kwashiorkor or marasmus if the deficiency is predominantly protein or predominantly energy, respectively.

As discussed earlier, good sources of bioavailable zinc such as meat and fish are also good sources of protein. Thus, when an individual is suffering from protein deficiency or PEM, zinc deficiency may also be induced due to limited zinc intake. Conversely, an individual with zinc deficiency may experience inanition, or reduced food intake, which could subsequently compromise protein and energy intake and induce PEM.

Children with PEM often have reduced plasma and tissue concentrations of zinc (Golden and Golden, 1979; Atalay et al., 1989). For these children, zinc supplementation in addition to diet therapy with adequate protein, has been shown to normalize plasma zinc concentrations where diet alone did not (Hemalatha et al., 1993). Experimental work with rats revealed that animals fed a zinc adequate, low protein diet also had significantly lower serum zinc concentrations than controls (Glore et al., 1993). Other clinical features common to both zinc deficiency and PEM include anorexia, diarrhea, stunting of growth,

wasting, skin desquamation and ulceration, fragility and dyspigmentation of hair, and compromised immunity (Golden and Golden, 1979).

Immunity

Numerous organs and cell types work in cooperation to mount an immune response against foreign and invading pathogens. Details of the immune system and immune response, including cell mediated immunity have been reviewed (Roitt et al., 1998). T lymphocytes, part of cell mediated immunity, function in the recognition of antigen, and have other effector functions such as secretion of cytokines and cytotoxic killing (Roitt et al., 1998). All T lymphocytes have a cluster of differentiation 3 (CD3) receptor which serves as a marker to identify them and differentiate them from other cell types. This CD3 receptor, also referred to as the T cell receptor (TCR), is a multicomponent aggregate which has an $\alpha\beta$ heterodimer and associated CD3 polypeptides (Chu and Littman, 1994). In addition to CD3, mature T lymphocytes have numerous other CDs present on their surfaces which act in concert to facilitate cell to cell contact and interaction. The presence of different CD receptors further classify T lymphocytes into sub-populations. Two major sub-populations are those T lymphocytes displaying either a CD4 or a CD8 receptor, and are classified as T-helper cells or T-cytotoxic/suppressor cells, respectively. During the maturation process in the thymus, cells from the bone marrow mature from double negative $CD4^-CD8^-$, to double positive $CD4^+CD8^+$, and then single positive $CD3^+CD4^+$ or $CD3^+CD8^+$ cells.

Zinc and Immunity

Increased susceptibility to infectious disease and immune dysfunction are common features of zinc deficiency (Keen and Gershwin, 1990; King and Keen, 1999).

This is not surprising considering the role of zinc in cell division and growth, and that the immune system is one of high turnover and rapid cell proliferation. In addition, a high amount of zinc is normally found in white blood cells, 25 times that found in erythrocytes, highlighting the importance of zinc in cells of the immune system (Hansen et al., 1982).

Naturally occurring biological models which have proved valuable for the study of zinc deficiency are the A46 mutation in Dutch Friesian cattle and AE in humans. In both cases zinc is poorly absorbed in the gastrointestinal tract. Just after birth the mutant A46 cattle quickly show signs of alopecia, growth arrest, and severe susceptibility to infection (Hansen et al., 1982). Absence of zinc treatment will result in death usually due to infection whereas zinc supplementation brings complete remission (Hansen et al., 1982). These animals also have small involuted thymuses, and a hypoplastic lymphatic system with an increased number of large immature lymphocytes. Cell-mediated immunity is severely impaired whereas humoral immunity is relatively intact. Similarly in AE, infants display skin lesions, anorexia, growth retardation, and susceptibility to infection. Autopsy examination of children with AE have revealed small or absent thymuses, and low levels of thymic lymphocytes (Hansen et al., 1982). Atrophy of the thymus in both diseases has implications for cell mediated immunity since this is the organ in which maturation of T lymphocytes takes place.

Induction of zinc deficiency in experimental animals is another important biological model. One characteristic of zinc deficiency which could act as a confounding variable in these studies on zinc and zinc deficiency is hypogeusia and related anorexia or weight loss. An experimental pair-fed or pair-weighed control group fed a zinc adequate

diet has been used to differentiate the effects of weight loss from zinc deficiency per se. Studies using this type of control group have helped to clarify the role of zinc and the impact of zinc deficiency. Common features of zinc deficiency, here described, have been attributed to the absence of dietary zinc as opposed to reduced feed intake.

Zinc deficient animals experience susceptibility to infection, loss of body weight, thymic involution, reduction in lymphoid tissue weight, and lymphopenia (Fernandes et al., 1979; Fraker et al., 1977; Salvin & Rabin, 1984). Feeding a zinc deficient diet to young mice (4-8 weeks old) resulted in lower thymus weight than control or pair-fed mice by week two of feeding, as well as complete involution of the thymus after six weeks of feeding (Fernandes et al., 1979). Fernandes et al. (1979) and Fraker et al. (1977) also examined lymphocyte function of these mice by testing their ability to produce antibodies to sheep erythrocytes (SRBC), a T-cell dependent antigen. T lymphocytes from zinc deficient animals displayed a poor response versus control or pair-fed animals whereas B lymphocyte function was not affected. Fraker et al. (1977) concluded that T-helper cells are particularly affected by zinc deficiency.

A further study was carried out to assess whether T-cell helper malfunction could be repaired in zinc deficient mice by feeding a zinc-adequate diet (Fraker et al., 1978). Five week old A/J mice were fed either a zinc adequate diet (50 ppm zinc) or a zinc deficient diet (< 1ppm zinc) for 31 days, after which both groups received the zinc adequate diet. As expected, body and organ weights, including spleen and thymus of zinc deficient animals were significantly reduced compared to control at the end of 31 days. After 4 weeks on the zinc-adequate diet, body weight and organ weights of the formerly zinc deficient mice returned to that of controls. Immunization of mice with SRBC at

weeks 2 and 4 of the repletion phase revealed a similar response to that of controls, indicating recovery of T lymphocyte function (Fraker et al., 1978).

Similarly, the effects of zinc deficiency are also seen in cells from zinc deficient animals assessed via in vitro assays. Spleen and peripheral blood lymphocytes from zinc deficient rats have shown a significantly lower proliferative response to the T-cell mitogen concanavalin A (Con A) and phytohemagglutinin (PHA) than lymphocytes from zinc adequate controls (Gross et al., 1979). Splenocytes from zinc deficient mice have also displayed a reduced proliferative response to ConA versus controls (Bossuyt, 1998). This indicates a loss of functionality and compromised ability to proliferate by T lymphocytes from zinc deficient mice. In contrast, Cook-Mills and Fraker (1993) found that the proliferative response to Con A in splenocytes from zinc deficient young adult mice was not significantly different from splenocytes from pair-fed or control mice. They concluded that it is only the reduction in T lymphocyte numbers which causes immune dysfunction as opposed to a loss of functionality.

Protein-Energy Malnutrition and Immunity

Many of the alterations in immunity due to malnutrition (PEM) are similar to those observed in zinc deficiency. Thus, it is important to include a brief discussion regarding PEM and immune function. PEM affects cell mediated immunity by reducing the number of T lymphocytes in blood and secondary lymphoid organs, and by promoting atrophy of the thymus and secondary lymphoid organs (Woodward, 1992). Bhaskaram and Reddy (1974) reported a decrease in cell-mediated immune function in children with PEM as evidenced by the significantly lower number of T lymphocytes compared to controls, and the decreased proliferative response of these cells to PHA

versus controls. In another study, young Bolivian children hospitalized with PEM had thymuses $1/10^{th}$ the size of controls, a higher number of immature circulating lymphocytes, and a lower number of mature circulating T-lymphocytes versus controls (Parent et al., 1994).

Experimental studies have revealed involuted lymphoid organs and compromised cell-mediated immunity in animals with PEM. Woodward and Miller (1991) have shown that weanling mice fed a low protein diet (0.5%) for 14 days induced cell mediated immunodepression and a significantly reduced response to SRBC antigens versus control. Lepage (1997) also found that splenic tissue was affected in adult mice fed either a low protein diet (2% protein), representing protein malnutrition, or an energy restricted diet, representing energy or calorie malnutrition. Not only was spleen weight significantly lower than controls, the percentage of spleen weight per body weight was significantly reduced versus controls and indicated a true reduction in lymphoid tissue weight. Total splenocytes per spleen were also significantly lower in the restricted groups versus control (Lepage, 1997). In addition, the level of dietary zinc in this experiment was adequate at 30 ppm indicating that the lymphoid changes were due to the protein or energy deficit.

Much of the literature regarding PEM, zinc, and immunity, points to lymphocyte dysfunction, including a reduced proliferative ability of lymphocytes from PEM, and zinc deficient animals. The theory which will be examined in this thesis, as to how zinc may exert this effect, involves signal transduction. Thus, a general discussion regarding cellular signaling follows.

Cellular Signaling

In order for a cell to respond to changes in its environment the cell must be able to recognize these changes and respond appropriately. How cellular components are able to communicate with one another is referred to as "signal transduction". Typically, a hormone, growth factor or antigen binds to a transmembrane receptor, a receptor which spans the cell membrane, and initiates a specific signal transduction pathway within the cell. Upon binding, conformational or enzymatic changes may occur within the receptor leading to a series of further intracellular reactions. These intracellular reactions may be mediated by "second messengers" a term which refers to reaction products of the initial chemical reactions in the signal pathway. Ultimately this cascade of reactions is able to affect the nucleus which can then direct any cellular processes which need to be altered or maintained in response to the signal received.

Cellular Signaling in T lymphocytes

The cellular mechanisms by which T lymphocyte proliferation occurs and the signal transduction pathway which delivers this message of proliferation to the nucleus are being unraveled through active research. Numerous proteins and enzymes are implicated in the maze of reactions involved in signal transmission. A hypothetical model of the early events of signal transduction in T lymphocytes is shown in Figure 1.

As described earlier, a CD3 receptor identifies or "marks" a T lymphocyte and the presence of other CDs help to classify these cells into subpopulations and are indicative of the function of these cells. Upon stimulation of the transmembrane CD receptors cellular processes are affected. Of particular importance is the antigenic stimulation of the CD3 receptor of T lymphocytes (TCR/CD3) by an antigen presenting cell (B

lymphocyte or macrophage) which ultimately results in lymphokine production and secretion, cell differentiation, and proliferation of these cells (Chu and Littman, 1994; Gelfand, 1993). The TCR/CD3 complex does not act in isolation but other CDs are able to enhance the intercellular communication and intracellular signaling (Ledbetter et al., 1991; Mills et al., 1991). In fact CD4 and CD8 function as accessory molecules or coreceptors of the CD3/TCR complex to enhance signal transmission (Weil and Veillette, 1996). Early responses to stimulation of the TCR/CD3 complex include an intracellular increase in tyrosine kinase activity, tyrosine phosphorylation, activation of phosphatidylinositol specific phospholipase C (PLC), and translocation of protein kinase C (PKC). While this thesis will focus on PLC and PKC, a discussion of these other early responses to stimulation of T lymphocytes is appropriate.

Tyrosine phosphorylation, mediated by tyrosine kinases, is an important early step in the T lymphocyte signal transduction pathway prior to PLC and PKC activation and is highlighted by in vitro studies using protein tyrosine kinase inhibitors. Inhibitors herbimycin and genistein minimize the intracellular tyrosine phosphorylation and the PLC activation of T lymphocytes (Gelfand, 1993). As the TCR/CD3 complex does not have any intrinsic kinase activity of its own, studies have examined several protein tyrosine kinases to account for this increase in kinase activity observed with antigenic stimulation of T lymphocytes. One tyrosine kinase implicated in this pathway is $p56^{lck}$, a protein which is unique to lymphocytes (particularly T lymphocytes). $p56^{lck}$ is phosphorylated upon antigen stimulation to the TCR and has been found to be physically associated with the CD4 and CD8 receptors indicating a role for the protein in the early stages of signal transduction (Weil and Veilette, 1994). Additionally, expression of

$p56^{lck}$, evaluated by Western Immunoblotting, has been shown to be higher in zinc deficient mice versus controls (Lepage, 1997). Interestingly, an increase in the expression of $p56^{lck}$ was also found in energy restricted mice versus controls (Lepage, 1997). Analysis of this data also revealed a significant negative correlation between serum or femur zinc status and expression of $p56^{lck}$ indicating that animals with the poorest zinc status also had the highest expression of $p56^{lck}$.

In addition, $p56^{lck}$ is known to associate with PLCγ1 after stimulation of Jurkat cells, a leukemic human T cell line, based on coprecipitation experiments (Weber et al., 1992). This gives evidence that activation of PLC is one step in the signaling pathway of T lymphocytes. It is uncertain whether other signal transduction proteins, such as PLC and PKC, of T lymphocytes will be affected by zinc deficiency, as demonstrated with $p56^{lck}$.

Phospholipase C and its Role in Signal Transduction

The PLC enzyme is responsible for the breakdown of phospholipids into inositol phosphates and diacylglycerol (DAG). There are four known types of PLC including α, β, γ, and δ, all of which are single polypeptide units with molecular weights ranging from 62-154 kDa (Rhee, 1991). Within each type of enzyme there are several enzymes which are numerically designated i.e. PLCγ1 and PLCγ2. Significant homology also exists between members within each enzyme type and also between different types. In addition, it has been reported that PLC from *Bacillus cereus*, which is similar in function and structure to mammalian PLC, contains zinc. Thus, PLC is a putative zinc metalloenzyme containing three zinc ions in its active site (Hough et al., 1989; Hansen and Hough, 1993). It also appears that zinc is essential for activity of PLC as inactivation of the *B.*

cereus PLC by ethylenediaminetetraacetate (EDTA) was reversed with addition of Zn^{2+}, partially reversed with Mn^{2+}, and only a minimal change or reversal was observed in the presence of Mg^{2+}, Ca^{2+}, Al^{2+}, Co^{2+}, Fe^{2+}, and Fe^{3+} (no statistics were presented) (Ottolenghi, 1965).

While the role of PLC in the signaling pathway of T lymphocytes is not unique to this cell type, as is the case with $p56^{lck}$, PLC is nevertheless an important part of this pathway. In vitro studies have shown that upon stimulation of the TCR/CD3 complex the PLCγ1 form of PLC is rapidly phosphorylated on tyrosine residues (Granja et al., 1991; Park et al.,1991; Secrist et al., 1991). Park et al. (1991) examined Jurkat cells, a human leukemic T cell line, incubated them with [^{32}P]orthophosphate and stimulated these cells with OKT3, a monoclonal antibody to CD3. After cell lysis, monoclonal antibodies to either PLCγ1 or PLCβ1 were used to obtain immuno-precipitates upon which sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was carried out. Stimulated cells showed a two-fold increase in ^{32}P content in PLCγ1 samples, whereas there was no change in the amount of ^{32}P content in PLCβ1 samples between stimulated and non-stimulated cells. A time course of PLCγ1 phosphorylation was also performed such that cells were stimulated with OKT3 for different time intervals. Phosphorylation reached its peak (167% of the non-stimulated level) at 3 minutes of stimulation, after which the amount of phosphorylation declined (no statistics given) (Park et al., 1991).

Phosphorylation of the PLC enzyme is needed for PLC activity and the resulting release of second messengers. The messengers, DAG and inositol phosphates, function in the activation of PKC and intracellular Ca^{2+} mobilization, respectively (Park et al., 1991; Secrist et al., 1991).

Protein kinase C and its Role in Signal Transduction

PKC is a common enzyme in signal transduction pathways including T lymphocytes where stimulation of the TCR/CD3 results in activation and activity of the enzyme. PKC is a family of isoenzymes which vary in their structure, expression in different tissues, mode of activation and substrate specificity (Hug and Sarre, 1993). Once activated by phospholipids and DAGs (or phorbol esters), PKC is translocated from the cytosol to the cell membrane where it phosphorylates other target proteins on serine and threonine residues (Bell and Burns, 1991). Additionally, several of the isoenzymes (α, β, γ) are calcium dependent and are referred to as conventional PKCs, whereas the calcium independent isoforms (δ, ε, ξ, η, θ) are referred to as novel PKCs (Hug and Sarre, 1993). Several structural regions are considered variable and others are constant when comparing the members of the PKC family. Phospholipid and DAG (or phorbol ester) binding sites are in the NH_2-terminal regulatory domain of PKC as well as the calcium binding sites for conventional PKC types, whereas the catalytic activity of the enzyme is generated from the COOH-terminal end.

PKC is also a zinc metalloenzyme which contains four zinc atoms within the lipid binding, NH_2-domain of the enzyme (Hubbard et al., 1991; Quest et al., 1992). Cysteine rich binding sites may be important as zinc ligands for formation of structural domains such as zinc binuclear clusters, similar to the zinc finger motif (Quest et al., 1992; Hurley et al., 1997). The requirement of zinc for activation of the enzyme is highlighted by the use of metal chelators which chelate zinc from the system. Human peripheral blood lymphocytes stimulated by BMA 030, an antibody against the TCR/CD3, invoke the translocation of PKC to the membrane and highlight its place in the T lymphocyte signal

transduction pathway. When TPEN, which acts as a heavy metal chelator, is part of the system, translocation of PKC does not occur (Csermely et al., 1988). Thus, the removal of zinc from the system inhibits the translocation of PKC.

Conversely, Csermely et al. (1988) examined the effect of additional zinc in culture media and found an increased amount of PKC activity at zinc concentrations of 0.7-1.0 mM, and above a concentration of 2.0 mM PKC activity was inhibited. Similar findings were also revealed by Murakami et al. (1987).

Differences in tissue distribution of the PKC isoforms is evident as the γ form is found only in the central nervous system while the α, βI, βII, δ, ε, and ζ are distributed fairly ubiquitously and (Hug and Sarre, 1993). Within T lymphocytes, several PKC forms are expressed, although the α and β forms have received the most attention. Studies on human T lymphocytes have revealed that upon activation of the TCR/CD3 with monoclonal antibodies against the TCR/CD3, translocation of the α form of PKC occurs within 10 minutes whereas translocation of the β form only occurs after 90 minutes of stimulation (Szamel et al., 1993). This indicates that the α form may be the first form to be phosphorylated once PLC activity has increased and second messengers have been released.

Summary

Malnutrition in the form of zinc deficiency and PEM has numerous detrimental effects on both humans and animals. Zinc deficiency results in impaired immunity and T lymphocyte proliferation, increased susceptibility to infection, and involution of the thymus. One of the mechanisms which may be involved in these changes is alterations in

the signal transduction pathway of T lymphocytes. It is known that expression of the signal transduction protein, $p56^{lck}$, is elevated in zinc deficiency and malnutrition syndromes but it is uncertain whether such alterations occur with other signal transduction proteins such as PLC and PKC.

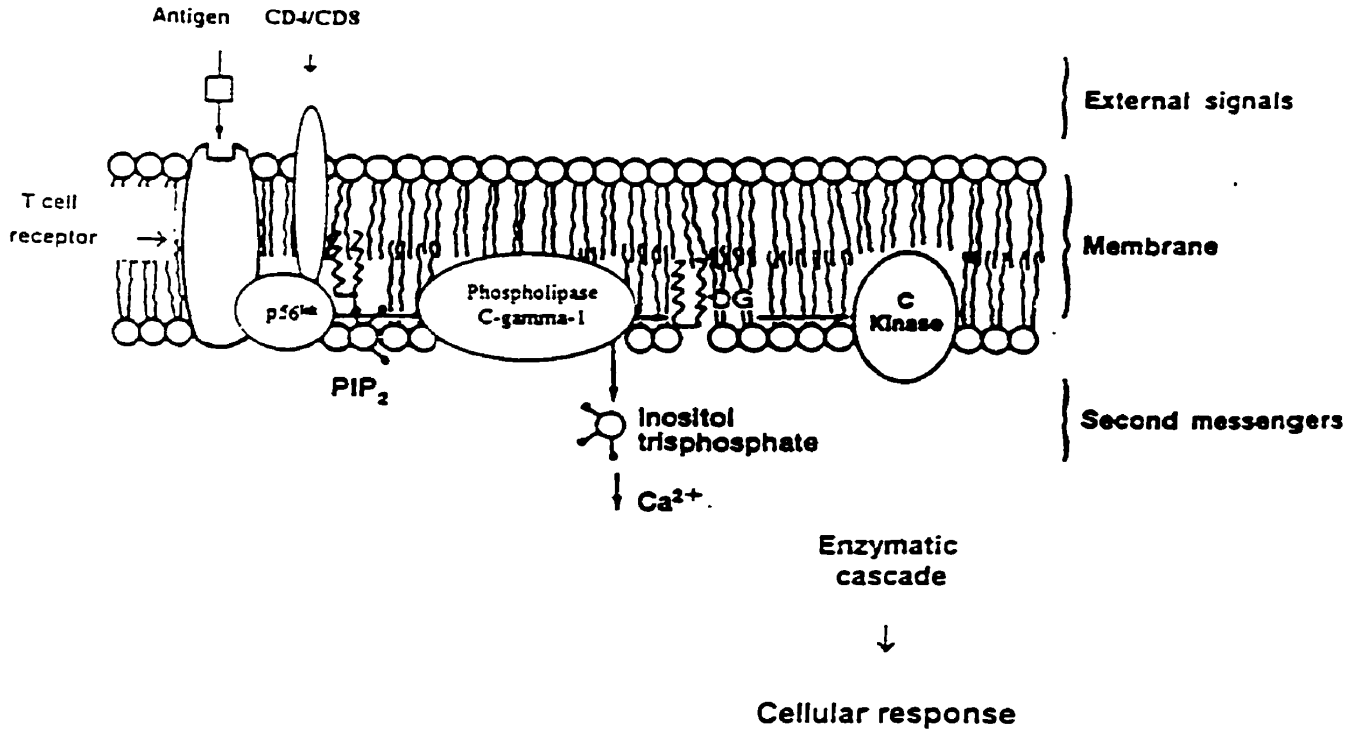

Figure 1. Early events in the T lymphocyte signal transduction pathway.
(Adapted from Ralws, 1987; Shankar and Prasad, 1998)

II. Study Rationale

Although zinc deficiency has received much study in the last several decades the underlying molecular and cellular mechanisms of immune dysfunction in zinc deficiency are largely unknown. The use of an animal model allows us to assess the in vivo effects of nutrient deficiencies and may offer a "truer" picture as to the mechanisms involved in human zinc deficiency. As zinc deficiency often occurs concomitantly with PEM it is important that studies not only examine the effects of zinc deficiency but also of PEM.

The present theory is that the detrimental effects of zinc deficiency on immune function may be mediated by an aberration of "zinc proteins" which require zinc for their structure or function. As cell mediated immunity is particularly affected in zinc deficiency, the study of T lymphocytes is of importance, particularly from splenic tissue as this organ contains a large number of mature T lymphocytes. The expression of an earlier T lymphocyte signal transduction protein $p56^{lck}$ is elevated in zinc deficiency and energy-type malnutrition compared to controls and therefore it is hypothesized that the T lymphocyte signal transduction proteins, PLC and PKC, may be up or down regulated in zinc deficiency thereby affecting signal transduction within T lymphocytes.

The objectives of the present studies were to assess the effects of zinc deficiency and malnutrition (energy, protein, or combined zinc and low protein) on animal weight, zinc status, spleen parameters and the splenic T lymphocyte signal transduction proteins: PLC and PKC.

In order to meet these objectives, immunologically mature young adult mice were subjected to dietary treatments including zinc deficiency, protein deficiency, zinc and protein deficiency, and energy restriction for 4 weeks. Mice were used in these studies as

an appropriate mouse model has already been established for this work, and the availability of antibodies which are cross reactive with mice. Young adult mice were used so as to avoid confounding variables such as growth and aging as would be seen in young and older mice, respectively. The dietary treatments used allow for examination of the effects of zinc, PEM, and a combination of zinc and PEM, in a time frame which is long enough to produce nutrient deficiencies but not result in animal death.

Technical advances and the widespread use of antibodies has enabled the advancement of many cellular and molecular techniques including the Western Immunoblotting and T lymphocyte selection process used in these experiments. Use of immunocolumns allowed the selection of T lymphocytes from spleen cell samples, and subsequent Western Immunoblotting on the T lymphocyte fraction.

Further characterization of the T lymphocyte signal transduction pathway and its requirement for zinc may help unravel the underlying mechanisms of immune dysfunction present in zinc deficiency and PEM.

III. Materials and Methods

Diet and Animal Model

Eight week old female C57BL/6 (Charles River Laboratories, St. Constant, PQ) mice were housed in an environment of controlled temperature (21-23 °C), humidity (55%), and light cycle (14 hr light / 10 hr dark) and fed a nutritionally complete standard chow ad libitum for 8 weeks. At 4 months of age, the mice were considered immunologically mature and were assigned to one of five dietary treatment groups for a period of 4 weeks: ZLP (1 ppm zinc + 2% protein), LP (2% protein), Z (1 ppm zinc), ER (energy restricted) or CTL (30 ppm zinc + 12% protein). The ER group was fed the same diet as the control group (30 ppm zinc + 12% protein), but in lesser quantities so as to attain body weights similar to the treatment which weighed the lowest, in this case, the LP and ZLP groups. In this way the ER group can serve as a control for the effect of weight loss commonly associated with protein malnutrition. All other diet treatments were fed ad libitum. Diet formulations, shown in Table 1, were based on the AIN-93M diet (Reeves, 1993). Diet ingredients were purchased from Harlan Teklad (Madison, WI), except for the dextrose (Moonshiners, Winnipeg, MB) and soy oil (Vita Health, Winnipeg, MB).

Due to time and space limitations needed to collect and process tissues at the end of the 4 week dietary treatment, animals started dietary treatment sequentially. To block for the effect of dietary treatment, one animal per dietary treatment group initiated treatment per day over an approximate 2 week time period. Upon commencement of dietary treatment, mice were transferred to hanging stainless steel cages so as to reduce

the possibility of zinc re-cycling. In this way waste products containing zinc would fall through the wire bottom of the cage and be caught in the wood shavings below. Animals had free access to distilled water which was provided in plastic bottles with stainless steel sipper tubes. Use of stainless steel reduced the possibility of zinc contamination which could be a problem with other forms of metal housing and water containers.

Animal weights were taken upon commencement of dietary treatment (baseline) and weekly thereafter, except for the ER group which was weighed more frequently. Animal care and handling was done in accordance with the protocol approved by the Local Animal Care Committee (University of Manitoba).

Tissue Collection

At the end of the 4 weeks of dietary treatment period mice were terminated by asphyxiation with CO_2 followed by cervical dislocation according to guidelines set out by the Canadian Council on Animal Care (Canadian Council on Animal Care, 1993). Animals were weighed and trunk blood was obtained after decapitation. Blood was placed on ice and subsequently centrifuged (1290 x g for 15 minutes at 4°C, Beckman Model TJ-6 centrifuge, Mississauga, ON) to separate the serum from the red blood cells. Serum was pipetted to fresh tubes and stored at -20°C. Spleens were removed using aseptic technique, weighed and processed immediately for separation of T lymphocytes. The hind legs of the animals were dissected, wrapped in foil and frozen at -20°C. Frozen serum and femur samples were analyzed for zinc at a later date.

Spleen Cell Isolation

All reagents were purchased from Sigma Chemical Company (St. Louis, MO), VWR Canlab, (Mississauga, ON), and Fisher Scientific (Nepean, ON) unless otherwise noted. Spleens were transferred to a sterile petri dish which contained 5 mls phosphate buffered saline (PBS) pH 7.3 (8.0 g/l NaCl, 0.2 g/l KCl, 1.44 g/l $Na_2HPO_4 \cdot (7\ H_2O)$, 0.24 g/l KH_2PO_4) with 2% bovine fetal calf serum (FCS) (Gibco, Grand Island, NY). Spleens were processed under sterile conditions in a Nuair biological culture hood (Plymouth, MN). Spleens were opened by making a small incision in one end of the spleen with a sterile needle and splenocytes were removed by running a bent needle along the spleen towards the incision so that cells would be forced out of the spleen cell sac. Once the majority of splenocytes were removed the spleen sac was discarded. The 5 ml spleen cell suspension was then transferred to a sterile 15 ml conical tube. A further 2 mls PBS/2% FCS was used to rinse the petri dish and was added to the conical tube which was then centrifuged at 300 x g for 5 minutes (Beckman, GS-6 centrifuge). The supernatant fraction was discarded and the pellet was resuspended in 2 mls Tris buffered ammonium (Tris-NH_4Cl) adjusted to pH 7.2 (working solution: 90 mls 0.16M NH_4Cl, 10 mls 0.17 M Tris pH 7.65) and gently inverted from end to end for 2 minutes in order to lyse red blood cells. Three mls PBS/2% FCS was then added and the suspension was centrifuged at 300 x g for 5 minutes. This was repeated 2-3 times so as to ensure lysis of all red blood cells. Cells were washed twice by resuspension in 8 mls PBS/2% FCS followed by centrifugation at 300 x g. Cells were resuspended in 3.5 mls PBS/2% FCS for cell counting.

Following the directions for use of the AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY) the total nucleated spleen cell count was determined. Ten µl of the cell suspension was added to 990 µl of 5% acetic acid in distilled water in order to lyse any remaining red blood cells. Ten µl of this solution was then loaded onto either side of the hemacytometer, cells were counted, and cell number calculated as follows:

$$\frac{\text{Average cell count x dilution factor x 10}}{\text{number of square mm counted}} \times \text{sample volume} = \text{total number of cells}$$

where dilution factor = 100, multiplication factor = 10 to account for a depth of 0.1 mm between grid and cover slip, number of squares counted = 4, and sample volume = 3.5 mls.

T Lymphocyte Isolation

Isolation of T lymphocytes from the spleen cell suspension was carried out with use of cellect™ Mouse T immunocolumns (Biotex Laboratories, Edmonton, AB), shown in Figure 2. Columns were set up according to the manufacturer's protocol in a Nuair biological culture hood (Plymouth, MN) to ensure a sterile environment. Each column was set up on a support and was washed with 20 mls PBS/2% FCS to produce an elutant which dripped out of the column at a rate of 6-8 drops per minute. The column reagent containing antibodies [polyclonal goat anti-mouse IgG (H+L)] was reconstituted with 1.5 mls PBS/2% FCS and used to activate the column at least one hour prior to use. These immunocolumns work on the basis of negative selection such that B lymphocytes are bound to antibodies in the column, allowing T lymphocytes to pass through and form the elutant. Prior to loading the sample, 20 mls PBS/2% FCS was used to wash the column.

The column was loaded with a maximum of 1 x 10^8 cells at a concentration of approximately 5 x 10^7 cells/ml, after which 20 mls PBS/2% FCS was used to wash the column of any remaining T lymphocytes. The T lymphocyte elutant was allowed to drip into two 15 ml conical tubes at a rate of 6-8 drops/minute. Samples were centrifuged at 300 x g for 10 minutes and the supernatant fraction discarded. The T lymphocytes were resuspended in 400 - 500 ul PBS/2% FCS for T cell counting; cells were then counted using the hemacytometer as was described earlier. Trypan blue exclusion was also performed to test the viability of cells. In this test clear cells are considered viable whereas coloured cells are nonviable as they have disturbed or broken membranes which has allowed entry of the trypan blue. At this point T cells were aliquoted to be frozen (at -80°C and used in enzyme activity assays) or were lysed in preparation for Western Immunoblotting.

The method described above for obtaining a pure T cell sample (>95% T lymphocytes) was verified by Lepage (1997) by flow cytometric analysis. For flow cytometry cells obtained from the T immunocolumn were stained with fluorescein anti-mouse T3 complex CD3ε (clone 145-2C11, Cedarlane Laboratories, Hornby, ON) and gated for the lymphocyte population using forward and side light scatter (Lepage, 1997).

T Lymphocyte Lysis

Samples for Western Immunoblotting were centrifuged at 400 x g for 5 minutes to pellet T lymphocytes and were then resuspended in 40 - 60 µls RIPA buffer [1% Nonidet P-40 (BDH Laboratory supplies, Poole, England), 0.25% deoxycholate, 150 mM NaCl, 50 mM Tris pH 7.5] containing protease inhibitors [1 mM sodium orthovandadate, 1 mM EDTA, 0.5% aprotinin, 12.5 µg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride

(PMSF)]. Lysed samples were vortexed and incubated in ice on an orbital rocker for 30 minutes. The sample was passed through a 27 G syringe to shear the DNA and microcentrifuged at 15600 x g (Eppendorf Centrifuge #5414) to separate the nuclear and cytosolic fractions. The supernatant (cytosolic) fraction was frozen in fresh tubes at -80°C until analyzed by Western Immunoblotting.

T Lymphocyte Protein Concentration

Prior to performing both Western Immunoblotting (PLCγ1 and PKCα) and enzyme activity assays (PLC and PKC) the protein concentration of samples was analyzed with the Bicinchoninic Acid (BCA) Protein Assay Reagent and protocol (Pierce, Rockford, IL or Sigma, St. Louis, MO). Protein standards were prepared from stock bovine serum albumin [2 mg/ml (Pierce, Rockford, IL) or 1 mg/ml (Sigma, St. Louis, MO)] and were prepared in appropriate buffer (PBS or RIPA for enzyme assays or lysates, respectively) to provide the following working range: 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml, and 1000 μg/ml. In triplicate, 10 μl of each protein standard, blank of appropriate buffer (PBS or RIPA), or diluted sample was pipetted into a 96 well plate. Two hundred μl of BCA working reagent was added to each well and the plate was mixed using the shaker on the microplate reader (Spectra Max 340, Molecular Devices, Sunnyvale, CA). The 96 well plate was incubated in the microplate reader at 37°C for 30 minutes and absorbances were read at 562 nm. The protein concentration of the samples was calculated using the software program on the microplate reader (SOFTmaxPRO, Version 1.2.0) as follows:

$$\frac{\text{Mean protein conc. (µg/ml) x assay volume (ml) x (dilution factor)}}{\text{sample volume (µl)}} = \text{µg protein/µl}$$

where total assay volume = 0.21 ml, dilution factor = 5, and sample volume = 10 µl.

PLCγ1 and PKCα Western Immunoblotting

To determine the expression of PLCγ1 and PKCα in murine T lymphocytes Western Immunoblotting was performed. Equipment and reagents were from Bio-Rad Canada (Mississauga, ON) unless otherwise indicated. Buffer formulations for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Immunoblotting were based on company protocols provided by Bio-Rad and Boehringer Mannheim (Laval, PQ). Ten µg of sample protein was solubilized by boiling in an equal volume of 2x SDS sample buffer [4% sodium dodecyl sulfate (SDS), 20% glycerol w/v, 10% β-mercaptoethanol (BDH Laboratory Supplies, Poole, England), 0.05% bromophenol blue, 0.5 M Tris pH 6.8] for 4 minutes. Samples, a positive control [either Non-Stimulated A431 Cell Lysate (Upstate Biotechnology, Lake Placid, NY) containing PLCγ1, or Rat Brain Lysate (Transduction Laboratories, Lexington, KY) containing PKCα], and a molecular weight ladder [7.1 - 208 kilodaltons (Kaleidoscope Prestained Standard, Bio-Rad)] were loaded onto a 5% stacking gel [1.4 ml H_2O, 0.33 ml 30% acrylamide mix, 0.25 ml 1.0 M Tris pH 6.8, 0.02 ml 10% SDS, 0.02 ml 10% ammonium persulfate (APS) made fresh daily, and 0.002 ml N,N,N',N' Tetra-methyl-ethylenediamine (TEMED)]. For SDS-PAGE, either a 10% separating gel for PLCγ1 (2 ml H_2O, 1.7 ml 30% acrylamide mix, 1.3 ml 1.5 M Tris pH 8.8, 0.05 ml 10% SDS, 0.05 ml 10% APS made fresh daily, and 0.002 ml TEMED) or an 8% separating gel for PKCα (2.3 ml H_2O, 1.3 ml 30% acrylamide mix, 1.3 ml 1.5 M Tris pH 8.8, 0.05 ml 10% SDS,

0.05 ml 10% APS made fresh daily, and 0.002 ml TEMED) was used. Electrophoresis was carried out in the Mini-Protean II electrophoresis cell with electrode buffer pH 8.3 (3 g/l Tris, 14.4 g/l glycine, 1 g/l SDS) at 170 V for approximately one hour. Separated proteins were then electrophoretically transferred to nitrocellulose membrane [0.45 micron (Bio-Rad)] using transfer buffer pH 8.3 (3.03 g/l Tris, 14.4 g/l glycine, 200 ml/l methanol) in a Mini Trans-Blot Electrophoretic Transfer Cell at 100 V for 1 hour. Before immunodetection, membranes were equilibrated with Tris buffered saline [TBS (6.05 g/l Tris, 8.76 g/l NaCl adjusted to pH 7.5 with HCl)].

Immunodetection of either PLCγ1 or PKCα was by chemiluminescence using the BM Chemiluminescence Western Blotting Kit for Mouse/Rabbit (Boehringer Mannheim). Membranes were incubated with a 1% blocking solution (concentrated blocking solution provided in the Boehringer Mannheim kit) overnight at 4°C to reduce nonspecific binding of antibody to the membrane. Antibodies used in immunodetection were anti-bovine PLCγ1 [mixed monoclonal of clones B-2-5, B-6-4, B-20-3, D-7-3, E-9-4 (Upstate Biotechnology, Lake Placid, NY) which has specificity primarily to PLCγ1, although it does react to some non-PLCγ1 SH-3 containing proteins, and is cross-reactive with bovine, rabbit, rat, mouse, and human species] and anti-human PKCα [clone 3 (Transduction Laboratories, Lexington, KY) which has specificity for PKCα but does cross react with PKCβ, and is cross-reactive with human, dog, rat, mouse, chicken, and frog species]. The antibodies were diluted in 0.5% blocking solution (1:1250 for anti-bovine PLCγ1 and 1:250 for anti-human PKCα) and membranes were probed for 1 hour at room temperature. Membranes were washed twice in TBS-Tween [TBS-T (1 ml Tween 20/l TBS)] and twice in 0.5% blocking solution; each wash was for 5 minutes.

The primary antibody was identified with an anti-mouse/rabbit IgG antibody linked to horseradish peroxidase (POD). This secondary antibody linked to POD was diluted in 0.5% blocking solution (1:2500 dilution) and allowed to probe the membrane for 1 hour at room temperature. The membrane was subsequently washed twice with TBS-T, for 15 minutes per wash, and was allowed to react with the luminescent substrate in the detection solution for approximately 1 minute. The light given off in this reaction was captured by photographic film exposure for approximately 3 minutes. Films were developed and visually inspected to ensure that the sample bands lined up with the positive control band. This indicated the presence of the same molecular weight protein in the sample as in the positive control. Films were subsequently scanned using a Relisys Reli9624 scanner and Adobe Photoshop (version 3) graphics software. Scanned images were analyzed by an image analyzer (IBAS II, Kontron Elektronik, Germany) which assigned arbitrary units to bands by accounting for the intensity and the size of the bands. For each film, bands were compared to and were assigned arbitrary units relative to the band representing CTL.

Membranes were also stained with India Ink to visualize the transfer efficiency of separated proteins from the gel to the membrane. Membranes were washed on an orbital shaker with Tween 20 solution (0.3% v/v in PBS) 3 times at 37° C for 30 minutes each, and twice at room temperature for 30 minutes each. Membranes were then stained with India Ink solution [0.1% v/v India Ink (Osmiroid International, Hampshire, UK) in Tween 20 solution] overnight at 4°C. Membranes were washed twice with Tween 20 solution until bands were visible against a lighter background and then air dried. Visual examination of the membranes revealed the approximate molecular weight of the protein

of interest by comparing the vertical location of the protein band with the molecular weight ladder.

To ensure that the addition of 10 μg of protein per well was appropriate, several Western Immunoblots were performed such that a range of protein amounts (5 - 25 μg for PKCα and 5 - 30 μg for PLCγ1) from one sample was loaded to each gel and processed as described for both PKCα and PLCγ1. This was important to examine as too much protein loaded onto the gel may saturate the reaction between protein and antibody such that no differences would be detectable beyond a certain amount of protein. As seen in Appendix A, immunoblots for both PKCα and PLCγ1 show that an increasing amount of either PKCα or PLCγ1 protein was detected as the amount of total protein added to the gel increased. This indicates that the addition of 10 μg of protein per well, as used with experimental samples, was appropriate as this amount was on the low end of the detection range and there is little danger of the protein-antibody reaction being saturated.

T Lymphocyte Protein Kinase C Activity

PKC activity in the isolated murine T lymphocytes was assayed with the protein kinase C enzyme assay system (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Whole T lymphocytes were used in this assay system, in which PKC catalyzes the transfer of the γ-phosphate group of [^{32}P] labeled adenosine-5'-triphosphate to a peptide specific for PKC. The amount of [^{32}P] incorporated into the peptide is counted, and is a reflection of the activity of PKC. The assay kit used is specific for PKC but the activity represents all isoforms. Assays or kits for specific isoforms of PKC are not available.

Samples were diluted in a homogenization buffer [such that the final concentration including sample was 50 mM Tris HCl pH 7, 0.3% β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 50 μg/μl PMSF, 10 mM Benzamidine with protease inhibitors (1.25 μg/ml leupeptin, 0.05% aprotinin) to help inhibit protein degradation], and kept on ice. Assay system reagents were prepared according to the manufacturer's protocol. This included the component mixture, which was an equal mix of four reagents provided [calcium buffer, lipid, peptide buffer, and dithiothreitol (DTT) buffer], the magnesuim [^{32}P] ATP buffer containing 40 μCi/ml, and a stop reagent.

To help insure consistency each sample or blank was run in duplicate. Twenty five μl of sample or sample buffer (for blanks) was added to an equal volume of component mix in each tube. Magnesuim [^{32}P] ATP buffer was then added to the top of each tube such that the drop clung to the side of the tube. Reactions were started by centrifuging tubes simultaneously for three seconds in a microcentrifuge. Tubes were incubated in a water bath for 15 minutes at 37°C. Reactions were terminated with the addition of the stop reagent and centrifuging for 3 seconds as was done to start the reactions. Samples were mixed by pipette and 35 μl of the total 65 μl of reaction mixture was pipetted onto individual paper discs and dried. Paper discs were washed twice in 5% acetic acid, and transferred to scintillation vials to which scintillant (ScintiSafe Econo 1) was added. Each vial was counted for one minute in a Beckman LS 6000TA Beta Scintillation Counter (Beckman Instruments Inc., Fullerton, CA). Prior to running the assay on the actual samples different incubation times and volume combinations were used to try to optimize the cpm obtained. However, these modifications helped

minimally with low counts (cpm), and the original protocol from the assay kit was followed for analysis of experimental samples.

As part of the data processing, three calculations were performed as per the assay protocol to determine the pmols transferred from [^{32}P] ATP to the protein substrate.

1. Calculation of specific activity (R) of 1.2 mM magnesium [^{32}P] ATP.

$$R = \frac{\text{cpm per 5 µl Mg[}^{32}\text{P] ATP}}{6} \quad \text{cpm/nmol}$$

2. Calculation of total phosphate (T) transferred to peptide and endogenous proteins.

$$T = (\text{sample cpm} \times \frac{65\mu l}{35\mu l}) - \text{blanks cpm}$$

where 65 µl represents the total terminated volume

and 35 µl represents the volume spotted onto the binding paper

3. Calculation of pmols phosphate (P) transferred per minute.

$$P = \frac{T \times 1000}{I \times R} \quad \text{pmoles/minute}$$

where I represents incubation time (min)

T Lymphocyte Phospholipase C Activity

As part of the second experiment, the activity of phospholipase C was of interest. Since assay systems directly assessing the activity of PLC were not available it was decided to determine the levels of the main product of the PLC reaction, inositol trisphosphate, and thereby indirectly assess the functionality of the protein. The assay system used was the D-myo-inositol 1,4,5-trisphosphate (IP_3) [^{3}H] assay system (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Whole T lymphocytes were used in the assay such that the unknown amount of IP_3 within the experimental samples

competes with the [^{3}H] IP_3 for a limited amount of binding protein. As with the PKC assay, samples and blanks were run in duplicate and the average value used in the calculations.

Assay reagents were prepared as described and included: concentrated D-myo-inositol 1,4,5-trisphosphate used for the preparation of standards; tracer containing D-myo-[^{3}H] inositol 1,4,5-trisphosphate; bovine adrenal binding protein; and assay buffer containing 0.1 M Tris buffer at pH 9.0, 4 mM EDTA, and 4 mg/ml bovine serum albumin.

All tubes received 100 µl of assay buffer and 100 µl of diluted tracer. Tubes representing Total Counts (TC) received 200 µl of distilled deionized water, whereas the remaining tubes received 100 µl binding protein and 100 µl of either: distilled deionized water [zero binding tubes (Bo)]; appropriate standard; sample; or concentrated standard solution [non-specific binding tubes (NSB)]. When all tubes had appropriate reagents, tubes were vortexed and incubated on ice for 15 min. Tubes were then centrifuged at 2000 x g (IEC Micromax, International Equipment Company; supplied by Fisher Scientific, Nepean, ON) for 15 minutes (4°C) after which tubes were inverted and the supernatant fraction discarded. Care was taken to ensure that all of the supernatant fraction was removed before resuspending the pellet in 1 ml of water. Tubes were then incubated at room temperature for 15 minutes before being decanted into scintillation vials. After addition of scintillation fluid (ScintiSafe Econo 1), radioactivity was counted in a Beckman LS 6000TA Beta Scintillation Counter (Beckman Instruments Inc., Fullerton, CA). The concentration of IP_3 was calculated as follows:

1. Calculation of the percent bound to the binding protein, using the average value, for each standard or sample.

$$\%B/B_0 = \frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(B_0 \text{ cpm} - \text{NSB cpm})} \times 100$$

2. The $\%B/B_0$ for the standards were plotted against the standard concentrations on semi-log graph paper. The %B/B for the samples were plotted on the standard curve to determine the IP_3 concentration of the samples.

Zinc Analysis

Serum, femurs and diets were assessed for zinc concentration by atomic absorption spectrophotometry using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, ON). Zinc standards of known concentrations of zinc were used to generate a standard curve.

Serum zinc was directly assessed simply by diluting the sample with a known amount of distilled deionized water immediately prior to reading the sample.

To prepare femurs for analysis, femurs were thawed and scraped with a scalpel blade to rid them of any surrounding muscle or connective tissue. After wet weights were obtained femurs were dried at 85°C for 48 hours and dry weights were taken. The femurs were digested by wet ashing with nitric acid as previously described (Clegg et al.,1981). Each femur was placed in an acid-washed test tube prior to adding 1 ml 70% nitric acid (Mallinckrodt AR Select for trace element analysis, Mallinckrodt, Phillipsburg, NJ). Femurs were digested for a minimum of 2 hours at room temperature and then heated at 85°C for 48 hours in a dry bath heater (Temp-Block module heater, Lab-Line

Instruments, Inc., Melrose Park, IL). After cooling at room temperature, samples were diluted with distilled deionized water to 25 mls and analyzed by atomic absorption. Similarly, 0.5 g of each diet was digested with 2 mls nitric acid for a minimum of 2 hours at room temperature, and heated at 85°C at 48 hours. Diet samples were diluted to 25 mls with distilled deionized water and analyzed by atomic absorption.

Statistical Analysis

Plots of data sets did not show any pattern formation which would indicate a lack of normality. The overall effect of treatment was assessed by a one way ANOVA using the general linear models procedure (SAS software release 6.04, SAS Institute, Cary, NC). If there was a significant treatment effect, then Duncan's Multiple Range Test was performed to assess significant differences between means. The probability level at which differences were considered significant was $P < 0.05$. Correlation analysis (spleen weight versus body weight) was performed using Pearson's correlation coefficient.

Table 1

Diet Formulation (g/kg diet)[1]

Ingredient	ZLP[2]	LP	Z	CTL[3]
Dextrose	887.5	877.5	762.5	752.5
Egg white	25	25	150	150
Soybean oil	40	40	40	40
Mineral mix[4]	35	35	35	35
Vitamin mix[5]	10	10	10	10
Choline	2.5	2.5	2.5	2.5
Zinc premix[6]	0	10	0	10

[1]Diet ingredients were purchased from Harlan Teklad (Madison, WI) with the exception of soybean oil (Vita Health, Winnipeg, MB) and dextrose (Moonshiners, Winnipeg, MB).
[2]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, CTL = control.
[3]ER group was fed the CTL diet in restricted amounts.
[4]AIN-93 zinc free mineral mix (Harlan Teklad).
[5]AIN-93 vitamin mix (Harlan Teklad).
[6]Zinc premix (5.775 g zinc carbonate/1000 g dextrose).

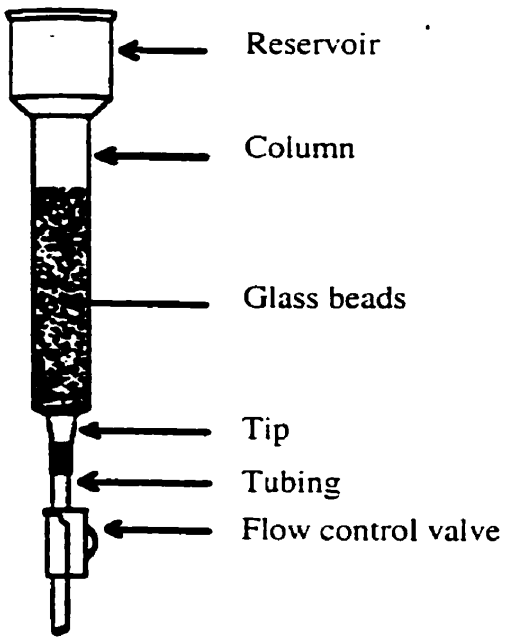

Figure 2. Diagram of cellect^{TM} Mouse T immunocolumn.

IV. Results

The results of the two experiments will be described separately for ease of description so as not to confuse the data sets.

Experiment 1

Body weight and zinc status

The primary measurements used in this experiment to indicate malnutrition and zinc status included weight loss and a change in serum and femur zinc status.

The initial weights of mice upon randomization to treatment groups did not differ among groups as shown in Figure 3. This figure graphically represents the weekly weights for mice in Experiment 1 and compares weekly weights among the treatment groups at each time point. At week 1, both LP and ER had significantly lower body weights than CTL. In addition, from week 2 until the end of the experiment, ZLP, LP, and ER had a significantly lower body weight than CTL. Importantly, from week 1 through to the completion of the experiment, the body weight of the ER group was not significantly different from the ZLP and LP groups indicating that the ER group would be appropriate to serve as a control for the effect of weight loss. Throughout the experiment only the Z group was not statistically different from CTL and at week 4 weighed 23.1 ± 0.7 grams versus 23.6 ± 0.7 grams, respectively.

Weekly weights were also examined for differences over time within each diet treatment, as shown in Table 2. It is evident that ZLP, LP, and Z groups experienced significant weight loss at week 1 compared to baseline or initial weight and that these body weights remained significantly lower for the remainder of the experiment.

Conversely, the Z group did not experience any significant weight changes throughout the experiment, whereas the CTL group had a significantly higher weight at weeks 3 and 4, compared to week 1.

Zinc status as determined by atomic absorption also differed among treatment groups as displayed in Table 3. Serum analysis revealed that the CTL and ER groups had the highest zinc concentration with 1.00 ± 0.07 μg/ml and 0.84 ± 0.06 μg /ml, respectively. The B, ZLP, and LP groups had significantly lower serum zinc levels compared to CTL (65%, 48%, and 52% of CTL, respectively) although these were not statistically different from one another. Serum zinc concentration in the Z group was 0.27 ± 0.04 μg/ml, significantly lower than the all groups including B and CTL such that the serum zinc concentration of the Z group was only 27% of that of the CTL group.

Femur zinc concentrations also varied among treatment groups. The CTL group had the highest zinc concentration which was 264 ± 8 μg/g dry weight femur. The B, LP, and ER groups had femur zinc concentrations not statistically different from CTL group. Femur zinc concentration was significantly lower in the zinc deficient groups, ZLP and Z, (84% and 80% of CTL, respectively) compared to the CTL group. In addition, ZLP and Z groups were not statistically different from each other or the LP group.

Spleen parameters

The effects of dietary treatment on splenic parameters of mice in Experiment 1 are given in Table 4. The B, Z, and CTL groups had the largest spleen weights (93.0 ± 3.9 mg, 96.2 ± 5.9 mg, and 97.1 ± 5.4 mg). The remaining three groups, ZLP, LP, and ER were not significantly different from each other but were significantly lower compared to the CTL (78%, 69%, and 76% of CTL, respectively) and B groups. Expressed as a

percentage of body weight, the only statistical difference found was between B and LP groups, with LP having a reduced weight compared to the B group. When compared to body weight, a positive correlation ($r = 0.81$, $p = 0.001$) was found between spleen weight and body weight (Figure 4). Regarding the number of splenocytes per spleen, the B and CTL groups had the most splenocytes/spleen ($13.8 \pm 0.8 \times 10^7$ cells and $13.7 \pm 1.2 \times 10^7$ cells, respectively). The LP and ER had significantly fewer spleen cells per spleen as compared to CTL (66% and 72% of CTL, respectively) and B groups. However, when the number of splenocytes were compared per mg spleen no significant differences were observed among groups.

PKCα expression and activity

The effect of dietary treatment on the expression of PKCα in murine T lymphocytes is shown in Figure 5. There were no statistically significant differences among the treatment groups and thus there was no effect of diet on the expression of PKCα.

To assess the effect of dietary treatment on the enzymatic activity of PKC the Protein kinase C assay system (Amersham) was used as described in the Methods section. As shown in Table 5, no significant differences were seen among the dietary treatment groups for PKC activity in splenic T lymphocytes.

Experiment 2

The second experiment followed the same experimental design as the first experiment with the main difference being that the protein of interest was PLC as opposed to PKC in the first experiment.

Body weight and zinc status

The initial weights of the mice were not statistically different from one another although the B group did have the highest mean weight at 25.2 ± 0.7 grams (Figure 6, Table 6). Figure 6 displays weekly weights and compares weights among treatment groups for a given time point. The ZLP and LP groups were significantly different from CTL by week 1 and remained significantly lower than CTL for the remainder of the experiment. The ER group was significantly lower than CTL at week 2 and week 4 time points. From week 1 to week 4 the Z group was not statistically different from the CTL group. By the completion of the four week treatment period, CTL and Z groups had the highest mean weights (24.2 ± 0.6 grams and 24.0 ± 0.4 grams, respectively). The remaining three treatment groups, ZLP, LP, and ER had reduced mean body weights compared to CTL and Z, however they were not statistically different from one another.

Weekly weights were also examined over the course of the experiment within each diet treatment group, shown in Table 6. Compared to baseline or initial weights, ZLP, LP and ER groups experienced a decrease in weight at week 1 through to completion of the experiment. Different from this pattern of weight loss are the stable weights of the Z and CTL groups. Weights within each of these groups did not differ significantly throughout the experiment.

Zinc status as measured by atomic absorption of serum and femur samples is reported in Table 7. Serum zinc concentrations were highest in the CTL, B, and ER groups (1.24 ± 0.19 μg/ml, 1.06 ± 0.03μg/ml, and 0.98 ± 0.08 μg/ml). The ZLP, LP and Z groups had a significant reduction in serum zinc concentration compared to the B, ER and CTL groups. The Z group had the lowest serum zinc concentration at 0.28 ± 0.19

µg/ml which represents only 23% of the CTL group. The serum zinc concentration of the Z group was not significantly different from the ZLP and LP groups.

In comparison with the amount of change seen with the serum zinc concentrations the effect of dietary treatment group on femur zinc concentration was evident in only one group. Femur zinc concentration was significantly lower in the Z group compared to the other treatment groups, including the ZLP and B groups. There was a 22% lower femur zinc concentration in the Z group compared to the CTL group (211 ± 11 µg/g dry weight femur and 269 ± 12 µg/g dry weight femur).

Spleen Parameters

As in Experiment 1, the spleen weights and related parameters of mice in Experiment 2 were calculated and are depicted in Table 8. The spleen weight of mice in the CTL group was 115 ± 8 mg. Spleen weights for the ZLP, LP, and ER groups were significantly lower than the Z, CTL and B groups but not statistically different from each other. This pattern of differences parallels the differences seen between groups regarding body weights. Figure 7 shows the positive correlation ($r = 0.60$, $p = 0.0002$) between spleen weight and body weight. To account for the differences in body weights, spleen weight was expressed as a percentage of body weight. Only the ER group had significantly lower percent spleen weight to body weight ratio compared to the CTL group. In addition, all treatment groups had a decreased number of splenocytes /spleen compared to the B group, including ER which was significantly lower than all groups except ZLP and LP. When splenocytes were expressed per mg spleen, only ZLP, ER and CTL were significantly lower than the B group.

PLCγ1 expression and activity

To assess the effect of dietary treatment on the protein expression of PLCγ1, western blotting was performed as described in the Methods section. There was no main effect of diet treatment on expression of PLCγ1. Figure 8 shows the arbitrary units for each treatment group and indicates the lack of any statistically significant differences among the groups.

To assess PLC activity, measurement of IP_3 in murine T lymphocytes was performed with the D-myo-Inositol 1,4,5-trisphosphate (IP_3) [^{3}H]assay system (Amersham). As shown in Table 9, no significant differences were found among the dietary treatment groups for PLC activity in splenic T lymphocytes.

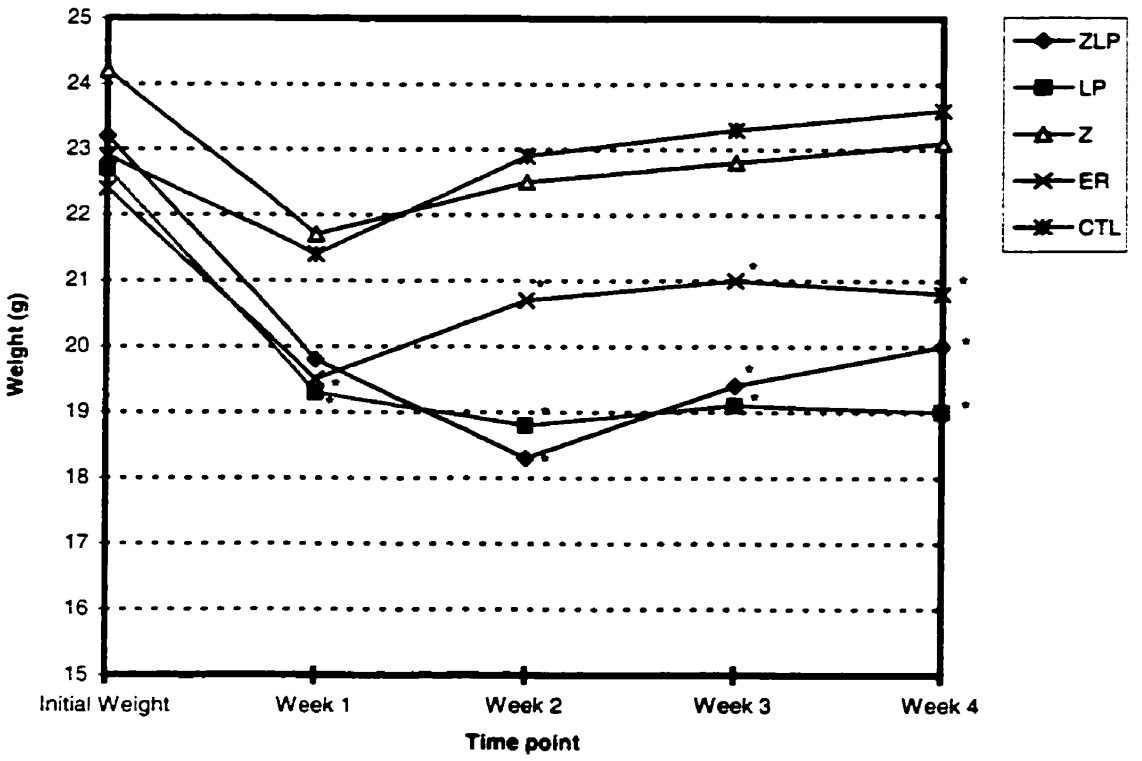

Figure 3. Effects of dietary treatment on weekly weights of mice in Experiment 1. Data points represent mean values for n = 7, except for LP, where n = 6 from weeks 1 - 4. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. An asterisk (*) denotes a significant difference from CTL for a given week. ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Figure 4. Scatter plot of spleen weight versus body weight of mice in Experiment 1. Data points are values obtained from individual mice, n = 34. Analysis by Pearson's correlation coefficient (r) revealed a significant positive correlation between spleen weight and body weight ($r = 0.81$, $p = 0.0001$).

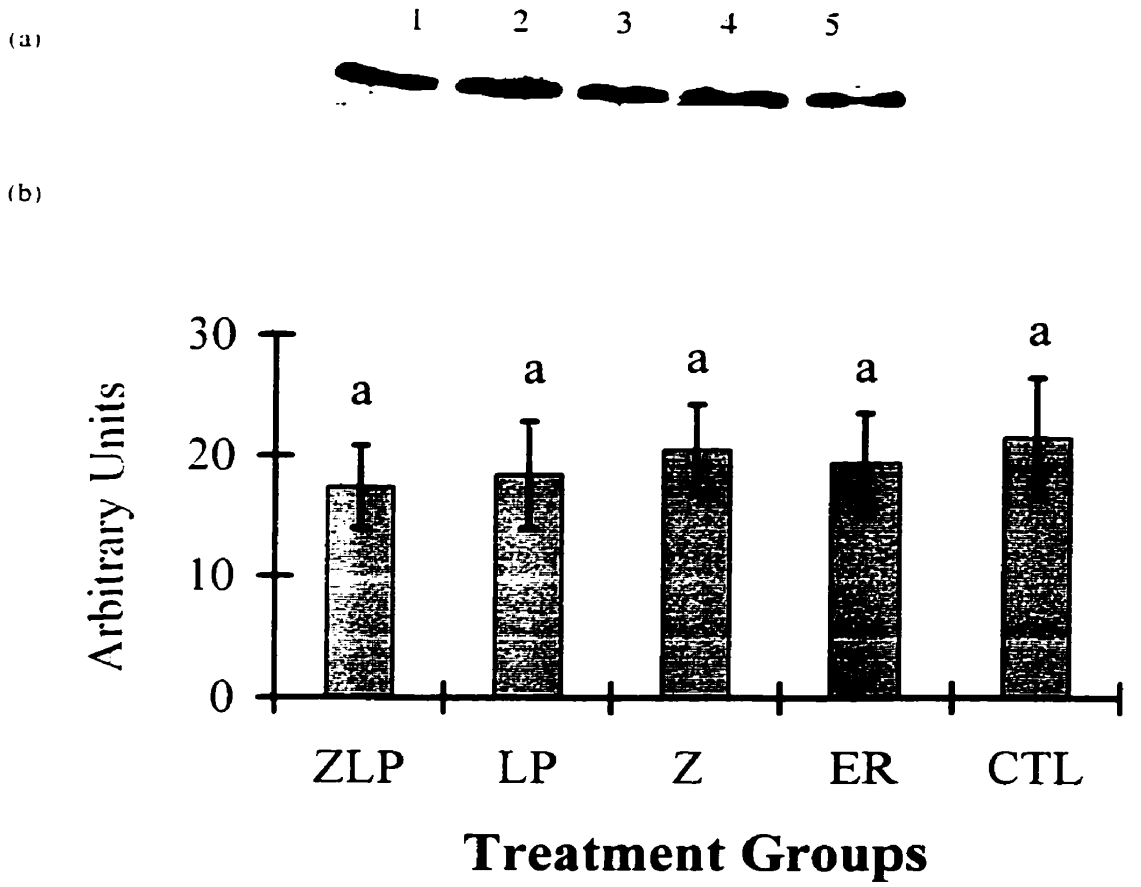

Figure 5. Effects dietary treatment on expression of PKCα in murine splenic T lymphocytes. (a) Representative Western immunoblot of PKCα expression in T lymphocyte lysates as detected by chemiluminescence. The dietary treatments were Zn deficient & 2% protein (ZLP, Lane 1), 2% protein (LP, Lane 2), Zn deficient (Z, Lane 3), energy restricted (ER, Lane 4) and control (CTL, Lane 5). (b) Arbitrary units for PKCα expression determined by image analysis scanning. Columns represent means ± the standard error of the mean, n = 7, except for ZLP and LP, where n = 6. The units represent arbitrary units relative to the CTL group.

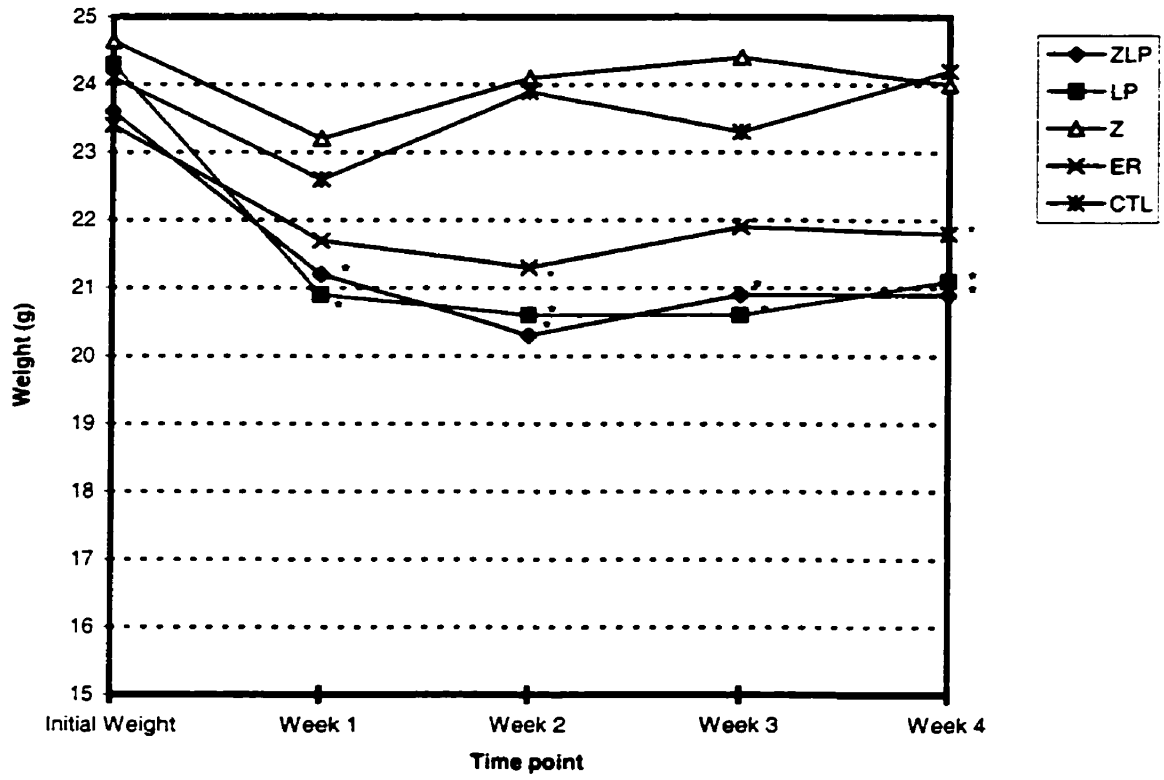

Figure 6. Effects of dietary treatment on weekly weights of mice in Experiment 2. Data points represent mean values for n = 7. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. An asterisk (*) denotes a significant difference from CTL for a given week. ZLP=Zn deficient & 2% protein, LP=2% protein, Z=Zn deficient, ER=energy restricted, CTL=control.

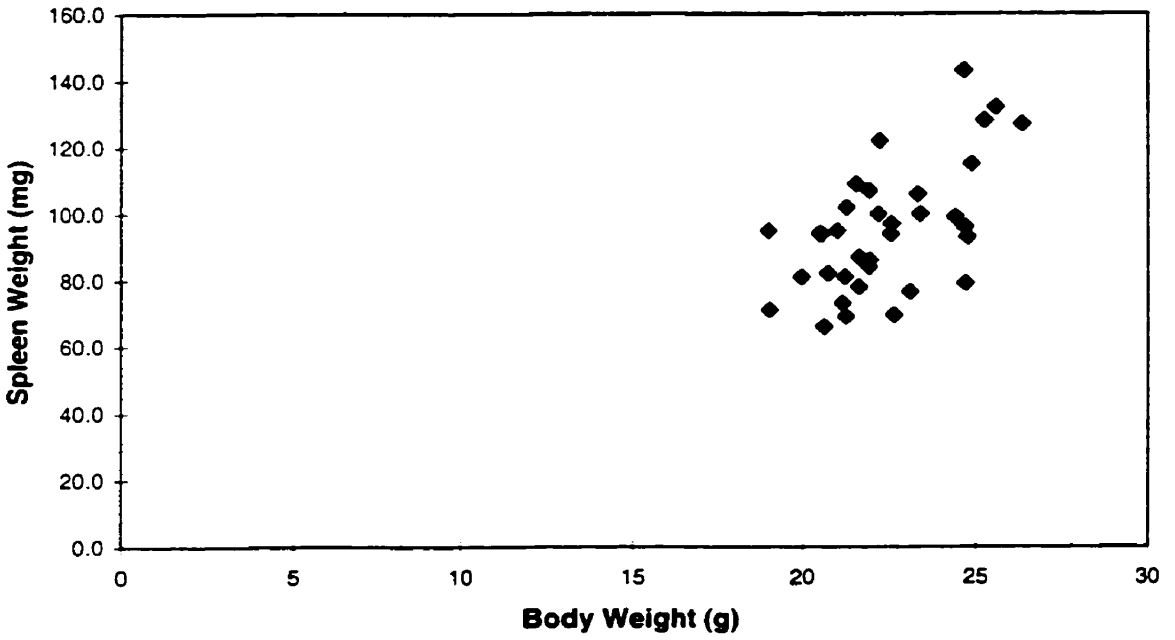

Figure 7. Scatter plot of spleen weight versus body weight of mice in Experiment 2. Data points are values obtained from individual mice, n = 35. Analysis by Pearson's correlation coefficient (r) revealed a significant positive correlation between spleen weight and body weight ($r = 0.60$, $p = 0.0002$).

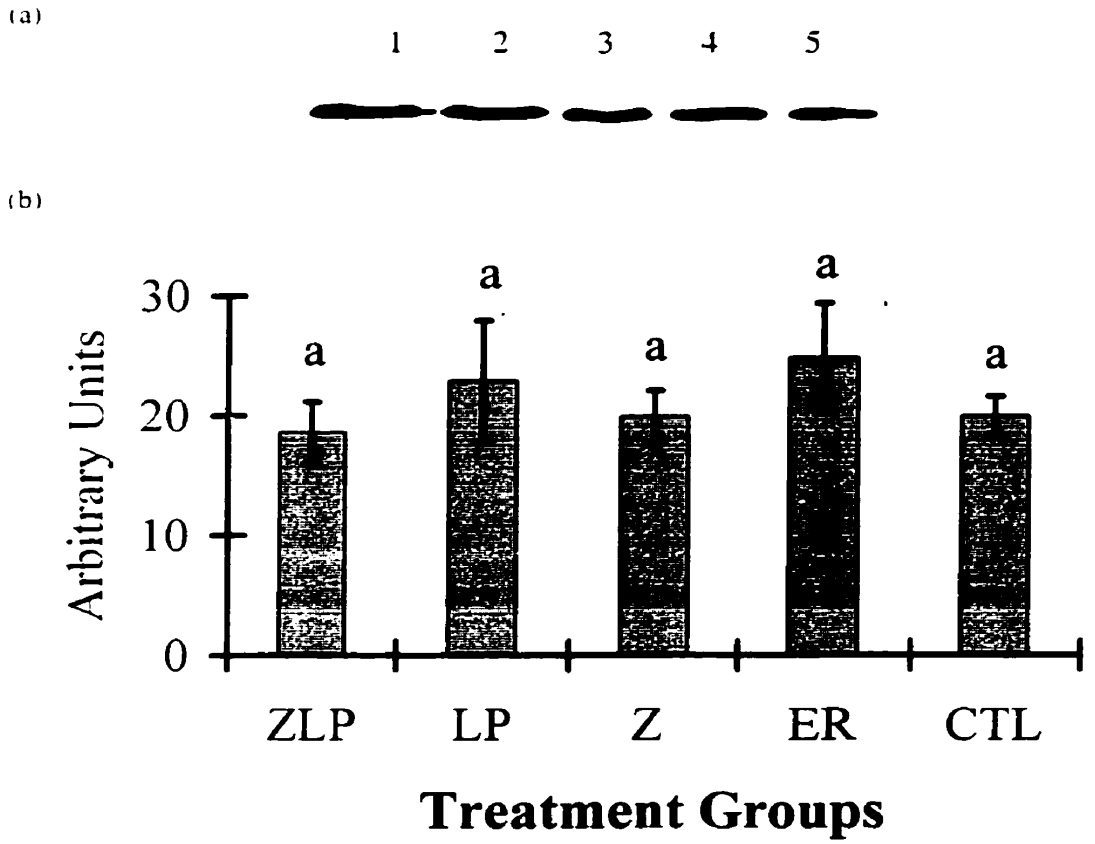

Figure 8. Effects of dietary treatment on expression of PLCγ1 in murine splenic T lymphocytes. (a) Representative Western immunoblot of PLCγ1 expression in T lymphocyte lysates as detected by chemiluminescence. The dietary treatments were Zn deficient & 2% protein (ZLP, Lane 1), 2% protein (LP, Lane 2), Zn deficient (Z, Lane 3), energy restricted (ER, Lane 4) and control (CTL, Lane 5). (b) Arbitrary units for PLCγ1 expression determined by image analysis scanning. Columns represent means ± the standard error of the mean for n = 7. The units represent arbitrary units relative to the CTL group.

Table 2

Effects of dietary treatment on body weight of mice in Experiment 1: Comparison within treatments[1]

	Treatment Groups[2]				
	ZLP	LP	Z	ER	CTL
Initial Weight[3]	23.2^{A} ± 0.8	22.7^{A} ± 0.5	24.2^{A} ± 0.7	22.4^{A} ± 0.4	$22.9^{A,B}$ ± 0.5
Week 1	19.8^{B} ± 0.5	19.3^{B} ± 0.4	21.7^{A} ± 0.8	19.5^{B} ± 0.7	21.4^{B} ± 0.4
Week 2	18.3^{B} ± 0.8	18.8^{B} ± 0.6	22.5^{A} ± 0.8	20.7^{B} ± 0.4	$22.9^{A,B}$ ± 0.5
Week 3	19.4^{B} ± 0.7	19.1^{B} ± 0.9	22.8^{A} ± 0.7	21.0^{B} ± 0.3	23.3^{A} ± 0.7
Week 4	20.0^{B} ± 0.6	19.0^{B} ± 1.0	23.1^{A} ± 0.7	20.8^{B} ± 0.3	23.6^{A} ± 0.7

[1]Values are expressed in grams as mean ± the standard error of the mean. For all groups n = 7, except for LP where n = 6 for weeks 1-4. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. For a specific diet treatment, different superscript letters indicate significant differences between weekly weight means as determined by Duncan's multiple range test.

[2]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

[3]Initial weights given for all treatment groups. Initial weight for the Baseline group was 22.1 ± 0.2 grams. Main effect of diet for initial weights, including the Baseline group, was not significant.

Table 3

Effects of dietary treatment on zinc status of mice in Experiment 1[1]

	Treatment Groups[2]					
	B	ZLP	LP	Z	ER	CTL
Serum zinc (μg/ml)	0.65^{B} ± 0.07	0.48^{B} ± 0.06	0.52^{B} ± 0.07	0.27^{C} ± 0.04	0.84^{A} ± 0.06	1.00^{A} ± 0.07
Femur zinc (μg/g dry weight)	256^{A} ± 8	223^{B} ± 4	$238^{A.B}$ ± 6	210^{B} ± 16	259^{A} ± 14	264^{A} ± 8

[1]Values are expressed as mean ± the standard error of the mean. For all groups n = 7, except for LP where n = 6. Main effects of diet were significant for serum zinc and femur zinc. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.
[2]B = baseline, ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Table 4

Effects of dietary treatment on splenic parameters of mice in Experiment 1[1]

	Treatment Groups[2]					
	B	ZLP	LP	Z	ER	CTL
Spleen weight (mg)	93.0^{A} ± 3.9	73.6^{B} ± 5.1	66.8^{B} ± 9.7	96.2^{A} ± 5.9	73.6^{B} ± 3.4	97.1^{A} ± 5.4
% Spleen/Body weight	0.42^{A} ± 0.02	$0.37^{A.B}$ ± 0.03	0.34^{B} ± 0.04	$0.41^{A.B}$ ± 0.02	$0.35^{A.B}$ ± 0.02	$0.41^{A.B}$ ± 0.02
Splenocytes/ spleen (x 10^{7})	13.8^{A} ± 0.8	$11.6^{A.B.C}$ ± 1.0	9.1^{C} ± 1.6	$12.9^{A.B}$ ± 1.4	$9.8^{B.C}$ ± 1.1	13.7^{A} ±1.2
Splenocytes/mg spleen (x 10^{6})	1.50^{A} ± 0.11	1.60^{A} ± 0.13	1.42^{A} ± 0.13	1.32^{A} ± 0.08	1.31^{A} ± 0.11	1.41^{A} ± 0.09

[1]Values are expressed as mean ± the standard error of the mean. For all groups n = 7, except for LP where n = 6. Main effect of diet was significant for spleen weight, spleen weight as a percent of body weight, and number of splenocytes per spleen. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]B = baseline, ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Table 5

Effects of dietary treatment on PKC activity of splenic murine T lymphocytes[1]

	Treatment Groups[2]					
	B	ZLP	LP	Z	ER	CTL
PKC activity (pmol/min/μg protein)	169 ± 10	232 ± 35	163 ± 24	218 ± 12	204 ± 36	178 ± 40

[1]Values are expressed as mean ± the standard error of the mean, n = 5 for all treatment groups. Main effect of diet was not significant for activity of PKC.
[2]B = baseline, ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Table 6

Effects of dietary treatment on body weight of mice in Experiment 2: Comparison within treatments[1]

	Treatment Groups[2]				
	ZLP	LP	Z	ER	CTL
Initial Weight[3]	23.6^{A} ± 0.4	24.3^{A} ± 0.6	24.6^{A} ± 0.5	23.4^{A} ± 0.5	24.1^{A} ± 0.6
Week 1	21.2^{B} ± 0.3	20.9^{B} ± 0.2	23.2^{A} ± 0.3	21.7^{B} ± 0.4	22.6^{A} ± 0.3
Week 2	20.3^{B} ± 0.5	20.6^{B} ± 0.5	24.1^{A} ± 0.4	21.3^{B} ± 0.3	23.9^{A} ± 0.3
Week 3	20.9^{B} ± 0.5	20.6^{B} ± 0.4	24.4^{A} ± 0.5	21.9^{B} ± 0.4	23.3^{A} ± 0.8
Week 4	20.9^{B} ± 0.4	21.1^{B} ± 0.5	24.0^{A} ± 0.5	21.8^{B} ± 0.4	24.2^{A} ± 0.6

[1]Values are expressed in grams as mean ± the standard error of the mean. For all groups n = 7. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. For a specific diet treatment, different superscript letters indicate significant differences between weekly weight means as determined by Duncan's multiple range test.

[2]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

[3]Initial weights given for all treatment groups. Initial weight for the Baseline group was 25.2 ± 0.7 grams. Main effect of diet for initial weights, including the Baseline group, was not significant.

Table 7

Effects of dietary treatment on zinc status of mice in Experiment 2[1]

	Treatment Groups[2]					
	B	ZLP	LP	Z	ER	CTL
Serum zinc[3] (μg/ml)	1.06^{A} ± 0.03	0.37^{B} ± 0.16	0.64^{B} ± 0.12	0.28^{B} ± 0.12	0.98^{A} ± 0.08	1.24^{A} ± 0.19
Femur zinc[4] (μg/g dry weight)	270^{A} ± 8	261^{A} ± 4	263^{A} ± 12	211^{B} ± 11	262^{A} ± 13	269^{A} ± 12

[1]Values are expressed as mean ± the standard error of the mean. Main effects of diet were significant for serum zinc and femur zinc. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.
[2]B = baseline, ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.
[3]n = 7 for all treatment groups.
[4]n = 7 except for LP and C, where n = 6, and ZLP and Z, where n = 5.

Table 8

Effects of dietary treatment on splenic parameters of mice in Experiment 2[1]

	Treatment Groups[2]					
	B	ZLP	LP	Z	ER	CTL
Spleen weight (mg)	112^{A} ± 5	88^{B} ± 5	85^{B} ± 4	104^{A} ± 5	84^{B} ± 6	115^{A} ± 8
% Spleen/Body weight	$0.45^{A,B}$ ± 0.02	$0.42^{A,B}$ ± 0.02	$0.40^{A,B}$ ± 0.02	$0.44^{A,B}$ ± 0.02	0.38^{B} ± 0.02	0.48^{A} ± 0.03
Splenocytes/ spleen ($x10^{7}$)	16.7^{A} ± 1.0	$10.6^{C,D}$ ± 0.6	$11.0^{B,C,D}$ ± 0.8	13.7^{B} ± 1.2	9.65^{D} ± 1.1	$12.9^{B,C}$ ± 0.9
Splenocytes/mg spleen ($x10^{6}$)	1.49^{A} ± 0.07	1.21^{B} ± 0.06	$1.29^{A,B}$ ± 0.05	$1.33^{A,B}$ ± 0.14	1.14^{B} ± 0.06	1.13^{B} ± 0.05

[1]Values are expressed as mean ± the standard error of the mean, n = 7 for all treatment groups. Main effect of diet was significant for spleen weight, spleen weight as a percent of body weight, and number of splenocytes per spleen. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]B = baseline ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Table 9

Effects of dietary treatment on PLC activity of splenic murine T lymphocytes[1]

	Treatment Groups[2]					
	B	ZLP	LP	Z	ER	CTL
PLC activity (pmol/mg protein)	4.03 ± 1.49	2.86 ± 0.53	2.58 ± 1.16	2.79 ± 1.12	13.4 ± 7.17	4.12 ± 1.27

[1]Values are expressed as mean ± the standard error of the mean. n = 3 for all treatment groups except for ZLP where n = 2. Main effect of diet was not significant for activity of PKC.
[2]B = baseline, ZLP = Zn deficient & 2 % protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

V. Discussion

The objectives of the present studies were to assess the effects of zinc deficiency and malnutrition (energy, protein, or combined zinc and low protein) on animal weight, zinc status, spleen parameters, and the splenic T lymphocyte signal transduction proteins: PLC and PKC. Salient findings include the lack of differences among treatment groups regarding the expression and activity of PLC and PKC in T lymphocytes. Pure dietary zinc deficiency was not associated with weight loss or immunological impairment when evaluating spleen cell numbers and % spleen weight per body weight. In addition, protein restriction reduced serum zinc status such that differences were not seen between mice with protein deficiency and a combined zinc and protein deficiency. In fact, Experiment 2 showed that protein restriction resulted in a similar level of serum zinc as found in mice with pure zinc deficiency. These findings will be further discussed in the following sections.

Effects of Zinc Deficiency and PEM on Body Weight

The mouse model in the present experiments has been previously shown in our lab to induce zinc deficiency (Z group), protein malnutrition (LP group), and energy malnutrition (ER group) (Lepage, 1997). These malnutrition types were evaluated by liver lipid and serum albumin concentrations, in the same diets and mouse model as in the present thesis (Lepage, 1997). In protein malnutrition, lipid concentrations in the liver often increase and serum albumin decreases (Edozien, 1968). Similarly, Lepage (1997) found that the lipid concentration in the liver was significantly higher in the ZLP and LP groups compared to CTL, whereas the Z and ER groups were not significantly different

from CTL. Serum albumin was decreased in the ZLP and LP groups versus CTL, whereas it was significantly higher in the Z and ER groups versus CTL (Lepage, 1997). The lack of indicators of protein deficiency, plus the weight loss observed in the Z and ER groups suggest that these groups experienced energy malnutrition. Thus, in the present experiments, the ZLP and LP groups would be characterized by protein-type malnutrition, and the Z and ER groups by energy-type malnutrition.

Experiment 1

Weight loss was present in all deficient groups, except the Z group, and was evident at week one of the experiment (Table 2). This initial weight loss has also been observed in previous studies from our lab and may partially be due to the change over from eating laboratory chow (pellet form) to a powdered diet, and the change in type of housing (from shoebox housing with wood shavings to hanging cages). When Bossuyt (1998) acclimatized animals to a "powdered" chow diet and hanging cages, all groups experienced weight loss, highlighting the effect of these environmental changes. However, if this was the primary reason for the initial weight loss in the present studies, all groups including Z and CTL would have experienced this weight loss.

Body weights at completion of the four week treatment period were significantly lower in all the deficient groups compared to CTL, except for the Z group (Figure 3). The LP group had the lowest weight compared to CTL (20% less than CTL), which is a similar finding to two other studies using the same animal model (Lepage, 1997; Bossuyt, 1998). The ZLP group did not differ significantly from the LP group at week 4 indicating that the severity of malnutrition was not any greater with a "double deficiency"

(combined deficiencies of the macronutrient protein and micronutrient zinc) than a deficiency of only protein.

Previously in our lab, decreased weight was observed for zinc deficient adult mice compared to CTL (Lepage, 1997; Bossuyt, 1998). Thus, the absence of a significant weight loss in the Z group or a significantly lower body weight versus CTL is noteworthy. Zinc deficiency has been shown to be responsible for weight loss of animals in numerous other studies, although most commonly weanling animals have been studied (King et al., 1991; Luecke et al., 1978,). Use of weanling animals compounds the severity of the zinc deficiency by imposing the additional requirements of growth on the metabolic system as zinc is essential for growth. With this in mind, it would be expected that any weight loss due to zinc deficiency in an adult model would not be as severe as that observed in a weanling model. Inanition does not appear to have occurred with the adult murine model used in this experiment.

At completion of the experiment, the two protein deficient groups, LP and ZLP, had significantly lower weights compared to their initial weights and the final body weight of the CTL group. Expressed as a percentage, the weights of the LP and ZLP groups at week 4 were 80% and 85% of CTL, respectively. The effect of a low protein diet on body weight has also been observed previously in our lab and by other researchers. Lepage (1997) and Bossuyt (1998) found that after a four week feeding trial, mice fed a 2% protein diet had significantly lower weights compared to controls. Using a different animal model, Glore and colleagues (1993) found that young rats fed a 3% protein diet over a three week period resulted in a 10% weight loss. With a more severe protein restriction, Taylor et al. (1997) found that young adult mice fed a 0.5% protein

diet over four weeks had a body weight of only 70% of control animal weight. Thus, from the studies described, protein restriction seems to have a strong influence on body weight.

Experiment 2

The pattern of body weights of animals in Experiment 2 were similar to that of Experiment 1. Specifically, all deficient groups except the Z group had weights significantly lower than CTL, whereas Z was not significantly different from CTL (Figure 6). ZLP, LP and ER also experienced an initial weight loss at the week 1 time point compared to their initial weights, in contrast to both the Z and CTL groups which did not experience this loss (Table 6). As in Experiment 1, the Z group did not seem to experience inanition which often accompanies zinc deficiency. Regarding protein deficiency, ZLP and LP groups body weights at week 4 were 89% and 87% of CTL, respectively, which is similar to Experiment 1 where ZLP and LP were 85% and 80% of CTL weight, respectively.

Effect of Dietary Treatment on Zinc Status

Malnutrition has detrimental effects on zinc status as reflected by both serum and femur zinc concentrations (Lepage, 1997). In the human population, there is no diagnostic marker of zinc deficiency, and this is problematic in assessing the prevalence and severity of this deficiency (Solomons, 1998). Serum zinc status is known to be affected by other conditions, such as stress, which make this a poor indicator of zinc deficiency. However, in controlled animal studies, it is accepted that serum zinc can be used for assessment of "uncomplicated zinc deficiency experiments" (Cousins, 1989, p.81). Furthermore, the assessment of femur zinc in animal studies gives a picture of the

impact of chronic zinc deficiency as bone zinc turnover occurs at a much slower rate than plasma zinc.

Experiments 1 and 2

In both experiments, ZLP and Z animals had significantly lower serum zinc concentrations than CTL, with concentrations ranging between 23% and 48% of CTL (Table 3 and Table 7). Similar findings have been shown previously, both from our lab and from others. Lepage (1997) and Bossuyt (1998) found serum zinc concentrations of zinc deficient mice to be reduced to 50%-52% and 46%-55% of controls, respectively. After feeding a zinc deficient diet to 6 week old mice for 30 days, Cook-Mills and Fraker (1993) found these animals to have serum zinc concentrations between 49% and 53% of controls.

Femur zinc concentration was significantly lower than CTL and B in both the ZLP and Z groups of Experiment 1 (Table 3) whereas only the Z group of Experiment 2 was found to be lower versus CTL or B (Table 7). Findings similar to the Experiment 1 data were also observed by Lepage (1997) who fed the same diets, as in the present studies, for 28 days. In contrast, Bossuyt (1998) again, using the same diets for 28 days found no differences among treatment groups. Thus, it seems such a feeding regimen of 28 days is not always sufficient to observe reductions in bone stores of zinc as seen in Experiment 1. It is also interesting that in Experiment 2 it was the ZLP group which had significantly higher femur zinc concentration than the Z group, a finding which Lepage (1997) also noted and queried whether there could be a protective effect of the "combined deficiency" versus the single zinc deficiency on femur zinc status.

In both Experiment 1 and Experiment 2, serum zinc concentration was found to be lower in the LP group versus CTL. This is in contrast to the ER group which experienced no reduction or lowering of serum zinc concentration compared to CTL. Similarly, Lepage (1997) and Bossuyt (1998) found serum zinc concentration to be significantly lower in LP adult mice versus controls, whereas the serum zinc concentration of energy restricted animals did not differ from controls. Thus, it seems that protein malnutrition may have a more detrimental effect on zinc status than energy malnutrition. In a different animal model, young rats fed a 3% protein diet for three weeks experienced a significant reduction in serum zinc concentrations compared to baseline and controls (Glore et al., 1993). Similarly, three week old rats fed a 2% protein diet for three weeks had lower serum and femur zinc concentrations than baseline and control groups (Prescod, 1998).

PEM has also been associated with poor serum zinc status in human populations. In marasmic children, some features of zinc deficiency such as growth stunting and skin ulcerations were associated with low plasma zinc concentrations (Golden and Golden, 1979).

A low protein diet or PEM may be able to exert this effect on serum zinc concentrations by several mechanisms. Although not measured in the present experiments, food intake may be lowered in protein deficient animals thereby decreasing zinc intake. Absorption may also be affected as protein and amino acids ligands, known to enhance absorption of zinc, are unavailable and as gut atrophy may also occur further reducing absorption (Atalay et al., 1989). Finally, plasma zinc transport proteins, such as albumin may be reduced in protein deficiency thereby reducing the zinc carrying capacity

of plasma (Hopkins, 1993). However, experimental studies from our lab have not supported the link between reduced serum zinc and albumin concentrations in protein deficiency (Lepage, 1997; Prescod, 1998).

Effect of Dietary Treatment on Spleen Parameters

While zinc deficiency and PEM have been examined for their effect on spleen parameters, few studies have examined these deficiencies in combination. Use of a combined deficiency treatment is important as low protein intakes are implicated in inadequate zinc intake in the human population (Sanstead, 1995). Thus, one of the advantages of the dietary treatments used in these studies is the use of the ZLP group.

Experiment 1

Spleen weights from all deficient groups, except Z, were lower versus CTL and B, whereas spleen weight of the Z group was not significantly different than CTL or B (Table 4). When body weight was taken into account, there were no differences among any of the treatment groups, indicating that the lower spleen weights observed in the ZLP, LP and ER groups may have resulted from the loss of body weight as opposed to a direct effect of the specific dietary treatment. This is also supported by positive correlation ($r = 0.81$, $p = 0.0001$) between body weight and spleen weight (Figure 4), again indicating that spleen weight may be a function of body weight. This finding is important as splenic atrophy is often attributed to zinc deficiency (Fraker et al., 1978; Fraker et al., 1986). Thus, the use of the treatment groups in the present studies, and lack of weight loss in the Z groups (Experiment 1 and 2) draw a different conclusion regarding splenic atrophy in zinc deficiency. When expressed as a percent of body weight, only the spleen weight of the LP group was significantly lower than the B group, revealing that the LP group was

the most severely affected treatment group in terms of spleen weight. The severity of the LP treatment was also demonstrated by Lepage (1997) who found the spleen weight of the LP group to be significantly lower than CTL when expressed as weight or as a percentage of body weight. Glore et al. (1993) also demonstrated a striking reduction in spleen weight of 33% when feeding young rats a restricted protein diet.

The number of splenocytes per spleen was lower in the LP and ER groups compared to B and CTL (Table 4). Interestingly, the zinc deficient groups were not significantly different from CTL or B groups, which again is presumably due to the lack of splenic atrophy which occurred with the other deficient treatment groups. The number of splenocytes per mg spleen was not significantly different among the B and treatment groups. Thus, for the LP and ER groups, the absolute number of splenocytes are significantly lower than CTL, but after accounting for spleen size, no differences were observed, indicating that reduced spleen weight is paralleled with a reduced cell number. Taylor and colleagues (1997) demonstrated that mice fed a 0.5% protein diet had a dramatically and significantly lower number of splenocytes per spleen versus controls (15% of control). In comparison, splenocyte numbers from the LP group of this experiment were not as severely depressed, presumably because the protein restriction of 2% was not as severe as the 0.5% restriction.

Experiment 2

Spleen weight data for Experiment 2 (Table 8) was similar to that of Experiment 1. The ZLP, LP and ER groups had significantly lower spleen weights than CTL, B, and Z groups. Only the ER group had a significantly lower spleen to body weight ratio than CTL in Experiment 2, whereas in Experiment 1, only LP was lower than B. Each of these

groups, ER (Experiment 2) and LP (Experiment 1), had the lowest spleen weight in absolute numbers in the respective Experiments, helping to explain why they are significantly lower when expressed as a percentage of body weight.

Results for splenocytes per spleen and splenocytes per mg spleen also differ between the two experiments. In Experiment 2, all treatment groups including CTL experienced a decrease in the number of splenocytes relative to the B group (Table 8). Similar findings were also reported by Lepage (1997). The largest reduction in the number of splenocytes per spleen occurred in the ER group which also displayed the lowest spleen weight and thus, corresponds with splenic atrophy. ZLP and LP groups were not significantly different from ER whereas the Z group had a significantly larger number of splenocytes per spleen than the ER group. This suggests that the protein restriction, with resultant weight loss, has a similar detrimental effect as energy malnutrition on reducing splenocyte numbers. These findings differ from other studies which have found a greater reduction in splenocyte counts in zinc deficient animals versus pair-fed controls (Cook-Mills and Fraker, 1993; Woodward and Miller, 1991). This is presumably due to the pattern of weight loss seen in other studies compared to the weight maintenance observed in these experiments. It is interesting to note that the pair-weighed controls, the ER group, in these experiments ultimately served as a control for the effects of weight loss in the ZLP and LP groups, as opposed to the anticipated weight loss of the Z group. Regarding the zinc deficient animals, the ZLP group had significantly fewer splenocytes per spleen than the Z group. This may indicate the more severe effect of the combined zinc and protein deficiency, or it may reflect the absence of weight loss in the Z group. Thus, a major difference in research findings concerns the

splenic parameters of the zinc deficient animals which are often found to be detrimentally affected by such dietary treatment. To account for spleen size, splenocytes were expressed per mg spleen; this data shows that all treatment groups were not significantly different from CTL and that the B group was significantly higher than CTL.

While the spleen parameters assessed in this thesis demonstrate splenic atrophy (associated with body weight) and some changes in the number of cells present, immunological function has not been assessed and is a limitation of the present studies. However, using the same animal model as in the present studies, Bossuyt (1998) revealed that Con-A stimulation of splenic T lymphocytes resulted in decreased proliferation in all four treatment groups (ZLP, LP, Z, and ER) compared to control mice.

Effect of Dietary Treatment on Signal Transduction Proteins

Upon initiation of the research project described in this thesis, it was hypothesized that the signal transduction proteins, PLC and PKC in T lymphocytes, would be negatively affected by zinc deficiency as these proteins require zinc for their structure and function (Hough et al., 1989; Hansen and Hough, 1993; Hubbard et al., 1991; Quest et al., 1992). As Experiment 1 was underway, Lepage (1997) revealed that the expression of an early signal transduction protein in T lymphocytes, $p56^{lck}$, was expressed at a higher level than controls. With this added information, it was realized that the expression or activity of proteins under investigation, namely PLC and PKC, from zinc deficient animals could also be higher than CTL.

Expression and Activity of PLC and PKC

PLC is one of the early signal transduction proteins in T lymphocytes, and as discussed earlier, upon stimulation of the TCR/CD3 complex, the PLC-γ1 form of PLC is

rapidly phosphorylated on tyrosine residues (Granja et al., 1991; Park et al., 1991; Secrist et al., 1991). PLC hydrolyzes PIP_2 into the second messengers, IP_3 and DAG, the latter of which activates PKC (Park et al., 1991; Secrist et al., 1991). In the light of elevated expression of $p56^{lck}$ in zinc deficient mice, it was thought that alteration of proteins PLC and PKC might also occur. This was not the case. Experiment 2 revealed that expression of PLCγ1 was not altered or different among the treatment groups (Figure 8) and that activity of PLC was not affected by any of the treatment groups (Table 9). Further to this, Experiment 1 showed no differences among dietary treatments regarding PKCα expression (Figure 5) and PKC activity (Table 5).

As Lepage (1997) revealed differences among Western Immunoblots using a sample size of n = 5 blots per treatment group, the use of more than 5 blots per treatment per group in the current experiments would seem adequate to be able to detect any meaningful differences among the treatment groups. Similarly, assessment of the activity of PKC in T lymphocytes (Experiment 1) would also seem adequate using an n = 5. Given the variation, or standard error of the mean, the activity of PKC (Table 5) among treatment groups does not appear to have any differences. In contrast, data from Experiment 2 regarding PLC activity was obtained from a small number of samples (n = 3 per treatment group, except for the ZLP group where n = 2). The limited amount of available sample was due to the small size of the murine spleen and consequently the T lymphocyte sample obtained from the immunocolumns is rather small. For each Experiment, the T lymphocyte sample was divided and aliquoted for Western Immunoblotting and activity assays, and "trial runs" in optimizing the procedure for the activity assays also required sample. Samples remaining for the experimental procedure

had to be pooled, ultimately reducing the "n" value per group. In addition, even though assay directives for the PLC assay were carefully followed, several parameters, such as Total Counts (radioactive counts), were different from the recommended even though several trials were performed. The experimental samples for PLC activity were still processed to assess feasibility of this system and any differences among the treatment groups. This data is still valuable as marked differences in the data were not observed, particularly when taking the variability or standard error of the means into account (Table 9).

Thus, from these experiments we can conclude that there were no significant differences among treatment groups for PLCγ1 and PKCα expression, or PLC and PKC activity. Although the reasons for this "no-effect" and the implications that these findings may have are uncertain, several theories will be examined.

Since zinc is required by both PLC and PKC and the expression and activity of these enzymes were not affected by nutrient deficiencies, it could be that zinc preferentially binds to these enzymes and remains bound even in deficiency conditions, whereas other enzymes may be more severely affected.

Changes could also occur at the gene transcription level such that decreasing zinc levels could cause a negative feedback mechanism to generate a basal amount of the enzyme.

Bossuyt (1998) showed that among the five dietary treatment groups (same as used in this experiment), there were no differences in splenic zinc content. This could imply that zinc content is highly conserved in splenic tissue even during zinc deficiency and thus a "no-effect" could be quite possible for protein within this environment.

Additionally, other nutrients could be involved in the maintenance of enzyme structure, expression and function when lower amounts of zinc are in the system. When PLC was inactivated in vitro by EDTA, zinc and to a lesser extent manganese, were shown to restore enzyme activity (Ottolenghi, 1965). A further experiment showed that after inactivation with EDTA, activity of PLC could be restored with zinc and cobalt and partially restored with manganese and magnesium (Little and Otnass, 1975). Thus, expression of PLCγ1 and activity of PLC could possibly be maintained by other divalent cations in the event of dietary zinc deficiency.

It has also been revealed that several nutrients may be able to modulate the activity of PKC. Antigen induced translocation of PKC to the plasma membrane can be inhibited by addition of a heavy metal chelator in in vitro systems. Reversal of this inhibition occurred with addition of zinc, and partial reversal was observed with iron and manganese (Csermely et al., 1988). Kuvibidila et al. (1999) have also shown that T lymphocytes from iron deficient mice have reduced proliferation in response to mitogen and reduced PKC activity compared to controls. This strengthens the argument that iron also has a key role in PKC functionality, and may be able to augment the role of zinc during a state of zinc deficiency. Thus while the crystal structure of PLC and PKC show zinc to be involved in their structure, other nutrients may be able to mimic or at least substitute for the effect of zinc.

Thus, from these experiments we can conclude that there were no significant differences among treatment groups for PLCγ1 and PKCα expression, or PLC and PKC activity. Although the reasons for this "no-effect" and the implications that these findings may have are uncertain, several theories will be examined.

As PLCγ1 and PKCα expression and PLC and PKC activity in T lymphocytes are not altered in T lymphocytes this may have implications for the importance of an earlier signal transduction protein, $p56^{lck}$. Lepage (1997) found that expression of $p56^{lck}$ was elevated in T lymphocytes in zinc deficiency, however, the exact meaning or mechanism for this response is unknown. As the activity of $p56^{lck}$ was not assessed by Lepage (1997) its level of function is uncertain. In the present experiment, an assay was attempted in order to assess the level of tyrosine kinase activity in T lymphocytes, and to give us some indication of the activity of $p56^{lck}$ (Appendix F). Although there was no detectable activity from this assay, a measurement of $p56^{lck}$ activity is still needed to help further understand the findings of Lepage (1997) and the current experiments. Future experiments could focus on the isolation of $p56^{lck}$ from CD3 stimulated cells from which a kinetic assay could be performed or assessment of the extent of phosphorylation at the $p56^{lck}$ autophosphorylation site (Tyrosine residue 394), which corresponds to $p56^{lck}$ activity, could be carried out. To complete the latter, Western Immunoblotting using an anti-phosphotyrosine antibody as a primary antibody would be appropriate. The major limitation hindering this research is the limited number of murine splenic T lymphocytes per animal.

Several studies have shown the detrimental effects of altered $p56^{lck}$ expression. Molina et al. (1992) found that $p56^{lck}$ deficient mice, generated by a *lck* null mutation, have thymic atrophy, few peripheral T lymphocytes, and no single positive thymocytes. Thus, thymocyte development and maturation seems to be altered as the normal progression of cells from double positive ($CD4^+/CD8^+$) to single positive ($CD4^+/CD8^-$ or $CD4^-/CD8^+$) was blocked. Transgenic mice overexpressing a catalytically inactive form

of $p56^{lck}$ are known to have decreased thymus size and reduced T lymphocyte counts (Levin et al., 1993). Further to this, Ericsson and Teh (1994) determined that the enzymatic activity of $p56^{lck}$ regulates positive selection of thymocytes during thymocyte maturation. It has also been shown that Jurkat T cells, transfected with an active form of $p56^{lck}$ were more susceptible to apoptosis with TCR stimulation versus a Jurkat cell line that was defective for $p56^{lck}$ expression and resistant to apoptosis (Di Somma et al., 1995). Together these studies indicate an important role of $p56^{lck}$ in thymocyte development and hence T lymphocyte production. In the context of the work of Lepage (1997) several possibilities arise. The elevated $p56^{lck}$ levels observed in zinc deficient splenic T lymphocytes could be a signal of cells undergoing apoptosis, and if so, this could account for the reduction in T lymphocyte numbers often seen in zinc deficiency. As higher amounts of immature T cells may be present in T lymphocyte populations from zinc deficient and PEM mice (Bossuyt, 1998), perhaps elevated $p56^{lck}$ levels serve as a marker of immature T lymphocytes. Finally, as PLC and PKC of splenic T lymphocytes do not seem to be affected by zinc deficiency this could imply that some signals of proliferation still continue, whereas in other cells once a threshold level of $p56^{lck}$ expression is exceeded these cells undergo apoptosis.

Since zinc is required by both PLC and PKC and the expression and activity of these enzymes were not affected by nutrient deficiencies, it could be that zinc preferentially binds to these enzymes and remains bound even in deficiency conditions, whereas other enzymes may be more severely affected.

Changes could also occur at the gene transcription level such that decreasing zinc levels could cause a negative feedback mechanism to generate a basal amount of the enzyme.

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during a state of zinc deficiency. Thus while the crystal structure of PLC and PKC show zinc to be involved in their structure, other nutrients may be able to mimic or at least substitute for the effect of zinc.

Finally, as numerous signaling pathways operate throughout T lymphocytes, in response to various stimuli via different receptors, expression and activity of PLC and PKC may not be altered due to its continued involvement in other intracellular pathways.

The findings of the present studies and the questions raised in this discussion have implications for future research directed towards understanding PLC and PKC. Functionality or expression of PLC and PKC in zinc deficient lymphocytes may be altered in cells stimulated by antigens to the TCR/CD3 complex, as opposed to the basal level examined in the present studies. It is also uncertain what effect zinc deficiency and malnutrition have on these proteins in different lymphocyte populations such as circulating T lymphocytes and whether any changes would also be seen in the human population. Future research examining any alterations in these early T lymphocyte signal transduction proteins may lead to discovery of an appropriate bio-marker for nutrition related immunodeficiency.

VI. Conclusions

General Conclusions:

- Dietary treatments ZLP, LP, and ER produced weight loss compared to initial weights and compared to CTL whereas pure zinc deficient mice (Z group) did not experience weight loss.
- Significant reductions in serum zinc was evident in the Z, ZLP, and LP groups compared to CTL. Femur zinc was also lower than CTL in the Z group (Experiment 1 and 2) and in the ZLP group (Experiment 1).
- Spleen weight was positively correlated with animal body weight.
- Expression of PLCγ1 and PKCα in murine splenic T lymphocytes was not altered in any of the treatment groups compared to CTL.
- Activity of PLC and PKC in murine splenic T lymphocytes was not altered in any of the treatment groups compared to CTL.

Future Research:

- More research is needed to reveal the specific molecular mechanisms by which zinc deficiency affects cell mediated immunity and the T lymphocyte signal transduction pathway.
- Investigations regarding PLC and PKC and the functionality of $p56^{lck}$ in zinc deficiency and malnutrition in T lymphocytes may lead to research in the human population regarding the use of these proteins as bio-markers of nutrition related immunocompromised status. An accurate indicator of zinc deficiency needs to be found so that zinc status can be effectively diagnosed and monitored.

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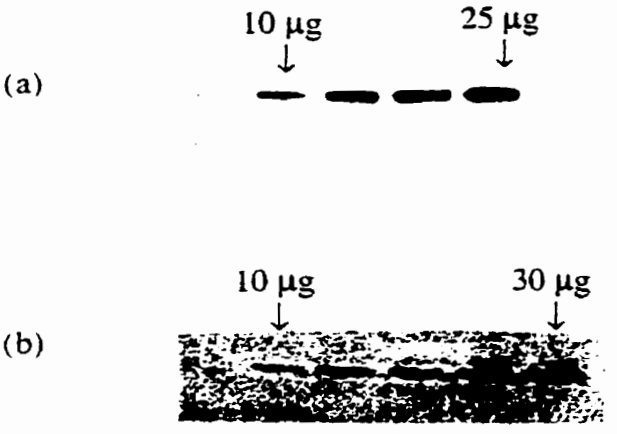
Appendix A



Linear range of PKCα (a) and PLCγ1 (b) in protein lysates from murine splenic T lymphocytes as detected by Western Immunoblotting. PKCα was detected in protein samples ranging from 5-25 µg protein (in 5 µg increments). PLCγ1 was detected in protein samples ranging from 5-30 µg protein (in 5 µg increments).

Appendix B[1]

Effects of dietary treatment on body weight of mice in Experiment 1: Comparison among treatments[2]

	Treatment Groups[3]				
	ZLP	LP	Z	ER	CTL
Initial Weight[4]	23.2^{A} ± 0.8	22.7^{A} ± 0.5	24.2^{A} ± 0.7	22.4^{A} ± 0.4	22.9^{A} ± 0.5
Week 1	$19.8^{B.C}$ ± 0.5	19.3^{C} ± 0.4	21.7^{A} ± 0.8	19.5^{C} ± 0.7	$21.4^{A.B}$ ± 0.4
Week 2	18.3^{C} ± 0.8	18.8^{C} ± 0.6	$22.5^{A.B}$ ± 0.8	$20.7^{B.C}$ ± 0.4	22.9^{A} ± 0.5
Week 3	19.4^{C} ± 0.7	19.1^{C} ± 0.9	$22.8^{A.B}$ ± 0.7	$21.0^{B.C}$ ± 0.3	23.3^{A} ± 0.7
Week 4	20.0^{B} ± 0.6	19.0^{B} ± 1.0	23.1^{A} ± 0.7	20.8^{B} ± 0.3	23.6^{A} ± 0.7

[1]Data corresponds to Figure 3.
[2]Values are expressed in grams as mean ± the standard error of the mean. For all groups n = 7, except for LP where n = 6 for weeks 1-4. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. For a specific time point, different superscript letters indicate significant differences between treatment means as determined by Duncan's multiple range test.
[3]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.
[4]Initial weights given for all treatment groups. Initial weight for the Baseline group was 22.1 ± 0.2 grams. Main effect of diet for initial weights, including the Baseline group, was not significant.

Appendix C[1]

Effects of dietary treatment on expression of PKCα in murine T lymphocytes[2]

	Treatment Groups[3]				
	ZLP	LP	Z	ER	CTL
Arbitrary units	17.4 ± 3.4	18.4 ± 4.4	20.5 ± 3.8	19.4 ± 4.2	21.5 ± 5.0

[1]Data corresponds to Figure 5b.
[2]Values are expressed as mean ± the standard error of the mean, n = 7, except for ZLP and LP, where n = 6. Main effect of diet was not significant for expression of PKCα.
[3]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Appendix D[1]

Effects of dietary treatment on body weight of mice in Experiment 2: Comparison among treatments[2]

	Treatment Groups[3]				
	ZLP	LP	Z	ER	CTL
Initial Weight[4]	23.6^{A} ± 0.4	24.3^{A} ± 0.6	24.6^{A} ± 0.5	23.4^{A} ± 0.5	24.1^{A} ± 0.6
Week 1	21.2^{C} ± 0.3	20.9^{C} ± 0.2	23.2^{A} ± 0.3	$21.7^{B.C}$ ± 0.4	$22.6^{A.B}$ ± 0.3
Week 2	20.3^{B} ± 0.5	20.6^{B} ± 0.5	24.1^{A} ± 0.4	$21.3^{B.C}$ ± 0.3	23.9^{A} ± 0.3
Week 3	20.9^{C} ± 0.5	20.6^{C} ± 0.4	24.4^{A} ± 0.5	$21.9^{B.C}$ ± 0.4	$23.3^{A.B}$ ± 0.8
Week 4	20.9^{B} ± 0.4	21.1^{B} ± 0.5	24.0^{A} ± 0.5	21.8^{B} ± 0.4	24.2^{A} ± 0.6

[1]Data corresponds to Figure 6.
[2]Values are expressed in grams as mean ± the standard error of the mean. For all groups n = 7. Significant main effects, as determined by repeated measures ANOVA, were week and diet x week. For a specific time point, different superscript letters indicate significant differences between treatment means as determined by Duncan's multiple range test.
[3]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.
[4]Initial weights given for all treatment groups. Initial weight for the Baseline group was 25.2 ± 0.7 grams. Main effect of diet for initial weights, including the Baseline group, was not significant.

Appendix E[1]

Effects of dietary treatment on expression of PLCγ1 in murine T lymphocytes[2]

	Treatment Groups[3]				
	ZLP	LP	Z	ER	CTL
Arbitrary units	18.6 ± 2.6	22.9 ± 5.0	19.9 ± 2.2	24.8 ± 4.6	20.0 ± 1.6

[1]Data corresponds to Figure 8b.
[2]Values are expressed as mean ± the standard error of the mean, n = 7 for all treatment groups. Main effect of diet was not significant for expression of PLCγ1.
[3]ZLP = Zn deficient & 2% protein, LP = 2% protein, Z = Zn deficient, ER = energy restricted, CTL = control.

Appendix F

T Lymphocyte Tyrosine Kinase Activity

To assess tyrosine kinase activity of T lymphocytes, which would provide information regarding the activity of $p56^{lck}$, the Protein Tyrosine Kinase Assay System (Life Technologies, Gaithersburg, MD) was used. The assay system provided a substrate buffer which contained a peptide substrate referred to as RR-SRC which contains a tyrosine phosphorylation site specific for tyrosine kinases. A control buffer was also provided which differed from the substrate buffer only in that it did not contain the substrate peptide. Prior to beginning the assay [γ-^{32}P] ATP was added to both the substrate and control buffer. In the assay system the tyrosine kinase present in the cell sample is able to phosphorylate tyrosine on the peptide substrate in the presence of [γ-^{32}P] ATP. Specifically, T lymphocyte samples were mixed with extraction buffer (50 mM HEPES, pH 7.4; 50 mM β-glycerophosphate; 25 mM sodium flouride; 0.1% Triton X-100; 150 mM NaCl; 20 mM EGTA; 15 mM magnesium chloride; 1 mM DTT; 25 μg/ml leupeptin; 25 μg/ml aprotinin) and incubated on ice for approximately 30 minutes. Samples were microcentrifuged at 15600 x g (Eppendorf Centrifuge #5414) to remove cellular debris and the supernate moved to fresh tubes on ice. Forty μl of each sample was divided equally into 4 microfuge tubes, after which 10 μl of the substrate buffer was added to 2 of the sample tubes, and 10 μl of the control buffer was added to each of the remaining 2 tubes as a control. Tubes were incubated for 30 minutes at 30°C after which reactions were stopped by adding 20 μl of ice cold 10% trichloroacetic acid. Samples were placed on ice for a minimum of 10 minutes and subsequently microcentrifuged at

15600 x g (Eppendorf Centrifuge #5414) for 10 minutes. Twenty µl of supernate (which contained the substrate peptide in the non-control tubes) were spotted onto phosphocellulose discs, which were washed twice with 1% acetic acid, and twice with distilled water. Discs were placed into scintillation vials, scintillation fluid (ScintiSafe Econo 1) was added, and radioactivity counted in a Beckman LS 6000TA Beta Scintillation Counter (Beckman Instruments Inc., Fullerton, CA). As radioactive counts for tubes with the substrate peptide were very low and similar to the control tubes (which did not contain the substrate protein) another trial was run in which additional sample was added to the tubes. However, protein tyrosine kinase activity was not detectable and it was decided not to further pursue this measurement.