EFFECTS OF DIETARY ZINC DEFICIENCY AND MALNUTRITION ON THE T-LYMPHOCYTE ZINC-FINGER PROTEIN $p56^{lck}$ IN MICE

by Lynne Lepage

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

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THE UNIVERSITY OF MANITOBA

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ABSTRACT

EFFECTS OF DIETARY ZINC DEFICIENCY AND MALNUTRITION ON THE T-LYMPHOCYTE ZINC-FINGER PROTEIN $p56^{lck}$ IN MICE

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Poor nutritional status increases the susceptibility to infection, which further compromises nutritional status, creating a vicious cycle of malnutrition and recurrent infection. Immune dysfunction is common in zinc deficiency, protein and energy malnutrition syndromes, however, the causative mechanisms are unknown. Investigations of zinc nutrition and immune defense have demonstrated that T-lymphocytes in particular are affected by zinc deficiency. The T-lymphocyte signal transduction pathway contains several zinc(Zn)-finger proteins in which the finger-like loop conformation is formed by Zn binding to cysteine sulfhydryl groups or histidine imidazole nitrogen groups. *In vitro* experiments have demonstrated that the function of Zn-finger proteins is adversely affected by zinc chelation and point mutations.

The objective of this experiment was to investigate the effects of dietary zinc deficiency (ZnDF) and malnutrition syndromes [2% protein deficiency (LP), combined Zn and 2% protein deficiencies (ZnDF+LP) and energy restriction (ER)] compared to the control group (C), on the type of malnutrition, zinc status, splenocyte counts and T-Lymphocyte expression of $p56^{lck}$, an early T-lymphocyte signal transduction Zn-finger protein. An adult murine model of zinc deficiency and malnutrition syndromes was developed in order to investigate these parameters.

Following a 4 wk feeding trial, mice receiving the ZnDF+LP, ZnDF, ER, and LP diets weighed significantly less than the C group, however, different forms of malnutrition were induced. Analysis of serum albumin and liver lipid concentrations indicated that protein-type malnutrition was induced in the ZnDF+LP and LP groups, and energy-type malnutrition was induced in the ZnDF and ER groups. Significant decreases in serum zinc and femur zinc pools were observed in the ZnDF+LP and ZnDF groups, indicating that zinc deficiency was induced in these groups compared to the ER, LP and C groups. A reductions in splenocyte counts was observed the ZnDF group ($1.56 \pm 0.06 \times 10^8$) compared to the C group ($1.91 \pm 0.13 \times 10^8$), however, the ER group ($1.44 \pm 0.03 \times 10^8$) experienced a decrease similar to that of the ZnDF group. For Western Immunoblotting of $p56^{lck}$, mouse splenic T-lymphocytes were isolated by immunocolumns. The expression of $p56^{lck}$ was elevated in all treatment groups compared with the control group. Surprisingly, the ZnDF+LP and the ZnDF groups had approximately a 1.7 fold increase in $p56^{lck}$ expression compared to the control group. Thus, it appears that dietary Zn deficiency may play a role in up-regulating the expression of $p56^{lck}$.

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I. Literature Review

Introduction

Malnutrition and its associated immunodeficiency syndromes are globally widespread and are responsible for approximately 15 million deaths annually (Beisel, 1994). Micronutrient deficiencies, occurring concomitantly with the more evident macronutrient deficiencies, are becoming increasingly recognized as important contributing factors in the development of malnutrition.

The micronutrient zinc plays an important role in immune function as exemplified by adverse immunological defects in zinc deficiency, and by reversal of these defects following zinc supplementation. This physiological requirement for zinc is understandable since zinc is an essential mineral that has numerous biochemical roles. Functional proteins that contain zinc are responsible for a broad range of biochemical functions, such as enzyme structure and activity, and antioxidant defense. Also, zinc is an integral component of plasma membranes. Not surprisingly, a broad range of physiological systems are affected by a deficiency of zinc, however tissues and cells of rapid turnover such as those involved in immune defense, are particularly affected.

Despite current knowledge of the functional roles of zinc in biochemistry and clinical manifestations of zinc deficiency, present knowledge of the mechanisms involved in the development of zinc deficiency pathology is limited. The purpose of this chapter is to present current knowledge of zinc deficiency pathology, particularly the effects of zinc deficiency on immune function. Previous hypotheses of the causative mechanisms of zinc

deficiency pathology will be reviewed. The major emphasis will be on the most current hypothesis: the role of zinc as an integral component of zinc-finger proteins using the T lymphocyte signal transduction pathway as the model of interest.

Nutrition and immune function

The immune system is an intricate network of specialized cells and organs that defend the body against invading pathogens. Details of the immune system and function have been reviewed (Abbas et al, 1991). A successful immune response, consisting of cellular interactions, signal production, transduction and cellular response, depends on the provision of an adequate supply of nutrients. Therefore, poor nutritional status can weaken host immune defenses and increase susceptibility to infection. In turn, infection can further compromise an already poor nutritional status, creating a vicious cycle between malnutrition and recurrent infection.

Prior to the recognition of the significance of micronutrient deficiencies, malnutrition syndromes were recognized as the primary culprits in nutrition-related immunodeficiency. Countless clinical observations and epidemiological studies have shown that various types of malnutrition such as protein deficiency (kwashiorkor), energy deficiency (marasmus) or protein-energy deficiency (kwashiorkor-marasmus) have adverse effects on all components of the immune system. However, cell-mediated immunity, a highly specific system involved with pathogen recognition and destruction, was particularly affected (Good et al, 1979)

Interestingly, when these various forms of malnutrition were produced in the laboratory with experimental animals, results were inconsistent with those from clinical

trials. In fact, cell mediated immunity was found to be normal or increased in animals receiving dietary restrictions (Hansen et al, 1982). These experimental findings led researchers to believe that other nutritional deficiencies, not accounted for in the laboratory might underlie this paradox (Hansen et al, 1982). This notion is conceivable since malnutrition is a complex web of multiple nutrient deficiencies, including a deficiency of micronutrients.

Zinc deficiency

i) Discovery of human dietary zinc deficiency

Prior to the 1960's, zinc deficiency seemed inconceivable due to the ubiquitous nature of zinc. Human dietary zinc deficiency was documented for the first time in adolescent Iranian males, suffering from growth retardation and delayed sexual maturity (Prasad et al, 1963). Analysis of their customary diet revealed the existence of a factor that impairs zinc absorption. Their diets consisted mainly of whole meal bread of high phytate content, which has been shown to act as an antagonistic ligand, rendering zinc less bioavailable for utilization by the body (O'Dell and Savage, 1960).

Further analysis of their dietary habits indicated that animal protein, known to be an excellent bioavailable source of zinc, was marginally consumed (Prasad et al, 1963). These initial observations indicated that zinc deficiency can be caused by high intakes of phytate containing foods, such as plant protein and cereal based diets, and also by the lack of adequate dietary zinc intake. Subsequent clinical studies of zinc supplementation in these populations have demonstrated the essentiality of zinc by reversal of their clinical symptoms (Sandstead et al, 1967).

Since the discovery of human dietary zinc deficiency, extensive investigations of other risk factors that may impair zinc absorption and metabolism have been pursued. Due to a better understanding of these factors, zinc deficiency has been identified in many conditions, and zinc has become recognized as a key nutrient in settings as complex as malnutrition and various disease states. As a result, zinc deficiency in humans is probably more widespread than first thought.

ii) Physiological conditions of altered zinc metabolism

Although maintenance of optimal nutritional status can play an important role in preventing serious medical complications, there are certain physiological conditions, unrelated to dietary factors, where zinc status may be adversely affected. Such conditions may be due to increased requirements, as seen with burns, malignancy and surgery, as well as increased zinc excretion observed in renal disease, muscular dystrophy, acquired immunodeficiency syndrome, diabetes and gastrointestinal infection (Cunnane, 1988).

iii) Interrelationship between malnutrition, infection and zinc deficiency

The etiology of zinc deficiency is diverse, since in addition to dietary factors (ie. inadequate zinc intake and decreased zinc bioavailability) zinc deficiency may also result from physiological conditions that affect zinc homeostasis. These include conditions of decreased absorption, increased requirements and increased excretion that may occur alone or in combination (Vallee and Falchuck, 1993). A discussion of zinc deficiency, as a component of malnutrition, provides an example of the involvement of dietary and physiological risk factors in the development of zinc deficiency.

Zinc deficiency is quite prevalent among the malnourished, and is commonly associated with immune dysfunction. Initially, inadequate intake of bioavailable zinc initiates zinc deficiency. Although inadequate intakes are known to increase the mineral's absorption in the gut, zinc absorption may be decreased due to changes in the intestinal mucosa brought about by malnutrition.

With the progression of malnutrition and decline in zinc status, susceptibility to infection is increased. Chronic infection can induce a hypercatabolic state where zinc requirements are increased. Meanwhile, nutritional status worsens. Furthermore, zinc excretion is increased in hypercatabolic states, due to zinc losses from tissue catabolism. Losses of zinc may also occur with gastrointestinal infection. This example clearly demonstrates that zinc deficiency can be of varied etiology and may be exacerbated by immune dysfunction secondary to zinc deficiency and malnutrition. As a result, a vicious cycle of declining nutritional status and chronic infection can be created.

iv) Onset of zinc deficiency: depletion of zinc pools and resulting clinical manifestations

As a result of altered zinc metabolism and inadequate compensatory dietary supply of zinc, homeostatic mechanisms may be unable to sufficiently provide zinc for maintaining biochemical functions, resulting in clinical manifestations of zinc deficiency. Contrary to other minerals, there does not appear to be a special storage site for zinc. However, various zinc pools are found within the body, with either rapid or slow phases of turnover (Cousins, 1996).

Serum and bone zinc pools will be discussed as they are relevant to this research project. Not only do these parameters serve to assess zinc status, but they are also

relevant in immune system investigations since immune cells (T lymphocytes) circulate in the plasma and the precursors to these cells originate in the bone marrow. Serum zinc (rapid turnover) representing approximately 1% of total body zinc, is accessible to all cells, however, it is quickly depleted with the onset of zinc deficiency (Vallee and Falchuk, 1993). In contrast, bone zinc pools are slowly mobilized. Although bone zinc pools contain 30% of body zinc, only limited amounts are mobilized since extensive mobilization of zinc from bone necessitates partial breakdown of this tissue (Ahou et al, 1993).

Other zinc pools of rapid turnover include the liver, pancreas, kidney, spleen and muscle, whereas the central nervous system is an example of a slow turnover pool. In any event, the onset of zinc deficiency and associated clinical manifestations may occur rapidly as little zinc is stored as reserve.

The clinical manifestations of zinc deficiency are diverse; anorexia, growth retardation, weight loss, dermatitis, night blindness and impaired immune function are among some of the features of zinc deficiency pathology (Table 1). Those afflicted may not suffer from all these clinical features as the severity of zinc deficiency syndromes is proportional to the extent of the deficiency. Individuals may display only certain clinical features, however most exhibit immune dysfunction (Fraker et al, 1986).

Zinc and immune function

The nutrient zinc and its immunoregulatory properties are now the focus of considerable interest. The interest in zinc and immune function stems from

Table 1 Features of Zinc deficiency

Anorexia

Impaired taste and smell

Weight loss

Loose frequent stools, malabsorption

Failure to thrive in infants and children

Delayed puberty, hypogonadism

Alopecia

Dermatitis and glossitis

Photophobia, night blindness

Neuropsychiatric symptoms, ataxia, dysarthria

Tremor, jitteriness

Increased susceptibility to infection, impaired immune function.

(Source: Aggett and Comerford, 1995)

observations of altered cell mediated immunity, generally manifested by increased susceptibility to infection in zinc deficiency (Hansen et al, 1982). The consequence of impaired cell mediated immunity can be serious since this branch of immunity is essential for providing highly specific protection against invading pathogens. This is accomplished by the involvement of T lymphocyte sub-populations. Helper T lymphocytes act in the coordination of the immune response by activating other immune cells and/or stimulating them to proliferate whereas cytotoxic T lymphocytes function by directly destroying their targets.

Evidence of impaired cell mediated immunity is provided by genetic malabsorption syndromes, which have proved to be useful biological models in assessing the effects of zinc deficiency on immune function. Reports of such syndromes in humans (acrodermatitis enteropathica) and in animals (mutant A46 Danish cattle), have suggested an association between zinc deficiency and immune dysfunction, since these conditions are characterized by frequent infection (Cunningham-Rundles and Cunningham-Rundles, 1988). Further analysis has revealed that susceptibility to infection corresponds to alterations in cell mediated immunity, as evidenced by depressed lymphocyte proliferation and delayed cutaneous hypersensitivity (DTH) in response to mitogen (Prasad, 1995; Moynahan, 1975). Both of these immunological parameters are indicative of cell mediated immune function; the former to assess proliferative capacity following antigen stimulation and the later to assess immune function in its ability to recognize and destroy foreign bacteria.

In these genetic models (acrodermatitis enteropathica and A46 mutation), immunological abnormalities could be corrected with adequate zinc supplementation, whereas the absence of supplementation most often results in death from infection in the A46 mutant cattle. In addition, upon postmortem examination, atrophy of the thymus was evidenced in both syndromes (Hansen, et al, 1982). The significance of this is that the thymus is a secondary lymphoid organ and site of maturation for T lymphocytes, the key players of cell mediated immunity. In the A46 mutant cattle, reductions in T lymphocyte numbers were observed, however, patients with acrodermatitis enteropathica displayed normal or decreased T lymphocyte numbers (Hansen et al 1982). Interestingly, although cell-mediated immunity was depressed, humoral (antibody) immunity in both conditions was not severely affected (Hansen et al, 1982).

Observations made from these naturally occurring zinc deficient models provide useful information about the biological importance of zinc for proper immune function, and in particular, for cell mediated immunity. Supporting evidence of these detrimental effects of zinc deficiency on immune function have been documented in human clinical trials and in experimental animals. Other zinc deficiency states in humans have been shown to result in immune dysfunction as seen by an increased susceptibility to pathogen invasion. Patients receiving long term total parenteral nutrition that subsequently developed zinc deficiency, displayed cell mediated immunological abnormalities such as a reduction in the numbers of circulating lymphocytes, and depressed T cell mitogenic response. Zinc supplementation was found to reverse these abnormalities (Keen and Gershwin, 1990).

Clinical studies of zinc deficiency, acquired as a consequence of protein, energy or protein-energy malnutrition have also demonstrated immune dysfunction. Golden and Golden (1979) reported a high incidence of infection in severely malnourished kwashiorkor, marasmic or kwashiorkor-marasmic Jamaican children with secondary zinc deficiency. Once again, the important role of zinc in immune function was demonstrated in this same study where zinc supplementation was found to improve immune response.

Impaired cell mediated immunity is also observed in rodent models of zinc deficiency (Keen and Gershwin, 1990). Numerous experimental studies have demonstrated that zinc deficient mice display impaired cell mediated immunity, as manifested by atrophy of the thymus and depressed delayed cutaneous hypersensitivity (Keen and Gershwin, 1990). Pathological analysis of zinc deficient animals revealed morphological changes in lymphoid organs, such as involution of the thymus and atrophy of the spleen (Keen and Gershwin, 1990). The lymphoid organs are those in which maturation, differentiation and proliferation of lymphocytes take place (Abbas et al, 1991). Since zinc deficiency has adverse effects on these organs, researchers have proposed that there may be alterations in the immune cells that they contain.

Dietary zinc deficiency in young A/J mice has been shown to result in reduced T helper function (Fraker et al, 1977). Since experimental zinc deficient animals display reduced dietary intake and weight loss, investigations of the role of weight loss on T helper function were warranted. Fraker et al (1977) conducted an additional experiment where pair feeding was used to control for decreased food intake of the zinc deficient dietary treatment group in young A/J mice. Contrary to zinc deficient animals, the energy

restricted animals did not experience a depression in T helper function. This observation indicates that zinc deficiency per se and not the weight loss that accompanies zinc deficiency has adverse effects on T helper function.

Several lines of evidence have shown that a depression of T helper function in zinc deficient mice corresponds to decreases in lymphocyte numbers. Fraker (1996), proposes that an immunodeficiency in zinc deficiency is due to a decrease in lymphocytes available to participate in the immune response, since residual lymphocytes were shown to function normally to mitogens (Cook-Mills and Fraker, 1993) and ratios of lymphocyte subpopulations were unchanged by dietary zinc deficiency (King and Fraker, 1991). Investigations as to the causes of lymphopenia associated with zinc deficiency are underway. Fraker (1996) proposes that zinc deficiency may cause lymphopenia by: 1) adversely affecting cell division, growth and proliferation, since zinc is known to be important for these cellular processes, and/or 2) increasing susceptibility of precursor B and T cells to apoptosis. To date, the causative mechanisms underlying immune dysfunction in zinc deficiency are unresolved.

Although zinc supplementation has been shown to restore immunological defects incurred by zinc deficiency, over-supplementation may have deleterious effects on host defense. Chandra (1984) demonstrated that excessive zinc supplementation (10 to 20 fold excess of the recommended dietary allowance) impaired immune responses in healthy adult men, as indicated by a reduction in lymphocyte stimulation response to phytohemaglutinin. Similarly, results of depressed immune responsiveness have been reported in experimental animals (Cunnane, 1988) . It appears that imbalances of zinc,

either deficiency or excess can have detrimental effects on immunity. Excessive intake of zinc supplements is discouraged, even though zinc has been shown to be important for immune function (Nutrition Recommendations, 1990). Recently, clinical trials have shown that the use of zinc lozenges may relieve the symptoms of the common cold more rapidly compared to a placebo group, however, adverse effects such as nausea and bad taste reactions were more common for those receiving zinc lozenges (Mossad et al, 1996).

Mechanisms underlying zinc deficiency pathology

Zinc is essential for diverse biological functions as demonstrated by the serious consequences of zinc deficiency. In order to explain this physiological requirement, and to elucidate the mechanisms underlying zinc deficiency pathology, researchers have explored and identified several biochemical roles for zinc.

The discovery of the requirement of zinc for the catalytic activity of carbonic anhydrase by Keilin and Mann (1940) was the first example of a functional role for zinc. Thereafter, over 300 enzymes have been shown to require zinc for catalytic function or structural stability (Vallee and Falchuck, 1993). Zinc metalloenzymes have various metabolic functions and are found among hydrolases, isomerases, ligases, oxidoreductases, and transferases. Despite this, evidence of an association between a deficiency of zinc, alterations in enzymatic activity and zinc deficiency pathology is lacking.

In addition to functional and structural roles in metalloenzymes, zinc is also thought to have a critical role in the stabilization of membranes. Bettger and O'Dell

(1981) hypothesized that zinc acts as a stabilizer in plasma membranes, as zinc is found in relatively high concentrations in membrane fractions. Also it is postulated that a deficiency in zinc disrupts membrane integrity by altering membrane fluidity, receptors and enzymes. In vitro studies have shown that a deficiency of zinc increases erythrocyte membrane fragility leading to cell death. However, the roles of zinc in plasma membranes in vivo remain to be elucidated, as experimental observations have shown only minimal changes in membrane structure during zinc deficiency (Bettger and O'Dell, 1993).

Zinc deficiency has been thought to increase susceptibility to free radical pathology. The presence of zinc in free radical defense metalloenzymes and metalloproteins suggests an indirect role for zinc in free radical defense (Willson, 1989). For example, zinc is a component of metallothionein, a sulfhydryl rich protein thought to be a good free radical scavenger and repair agent (Willson, 1989). Zinc is also a component of superoxide dismutase, a free radical destroying enzyme, however, Taylor et al (1988) reported that CuZn-superoxide dismutase activity in severely zinc deficient rats was not significantly altered, and that the primary free radical defense system was not severely compromised. To date, the mechanisms of zinc deficiency pathology have not been explained by the proposed theories based on the functional roles of zinc in enzymes, membranes and free radical defense. Exploration of other essential biochemical functions of zinc, such as its role in signal transduction, may help in uncovering this enigma.

The role of zinc in signal transduction

i) Zinc finger proteins

Signal transduction is a complex multistep process initially involving cellular activation, followed by integration and translation of the signal into a biological response. Zinc may play an important role in signal transduction since zinc is an integral component of zinc finger proteins, many of which are enzymes found in cellular signal transduction pathways (Cousins, 1996).

Zinc may exert its role by conferring structural stability to zinc finger proteins by the maintenance of the appropriate tertiary or quartenary structure. Furthermore, it has been suggested that zinc finger domains may serve to stabilize the specific binding of the zinc finger protein to another protein or to DNA (Klug and Schwabe, 1995). This property can be best described when considering zinc finger protein structure. Zinc finger-like loop domains are created by the binding of zinc in a tetrahedral formation with thiol groups of cysteine and/or imazadole nitrogen groups of histidine which creates a loop in the protein sequence (Figure 1).

The zinc finger motif was discovered in DNA binding domains of certain transcription factors in the cell nucleus (Miller et al, 1985). Subsequently, zinc finger proteins were shown to be widely distributed throughout the cell (Klug and Schwabe, 1995), suggesting their involvement in a broad range of cellular functions, including protein-protein interactions in signal transduction. Due to their widespread occurrence, zinc finger proteins may significantly contribute to the overall zinc requirement (Cousins, 1996). In vitro experiments have shown that zinc finger protein function is

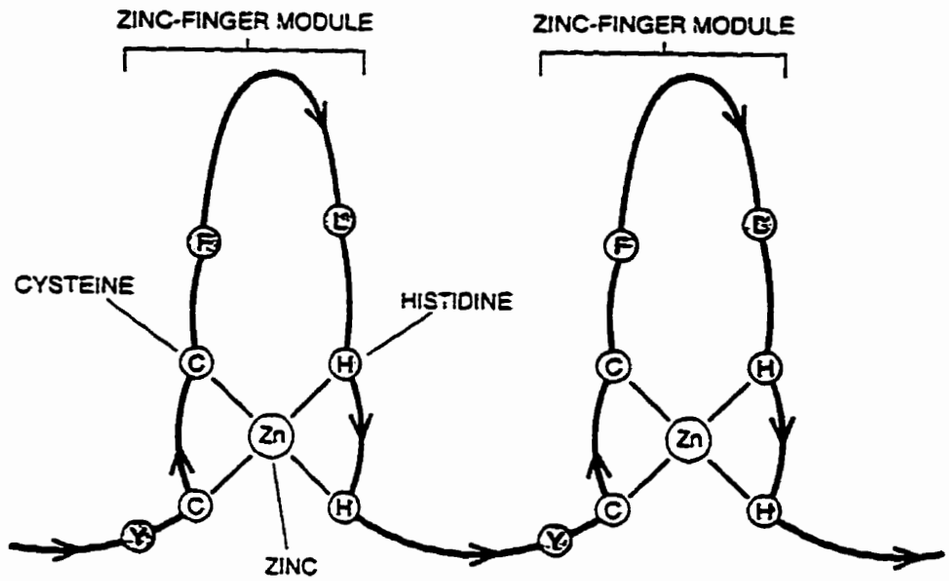

Figure 1: Molecular structure of zinc finger domains

Source: Rhodes and Klug (1993)

adversely affected by zinc chelation and point mutations (Pernelle et al, 1991; Turner et al, 1990). However, the effects of dietary zinc and/or protein deficiency on in vivo functioning of zinc finger proteins are unknown.

ii) T-lymphocyte signal transduction pathway: role of $p56^{lck}$

Since zinc deficiency can have profound effects on the immune system, particularly cell mediated immunity, an investigation into the effects of zinc deficiency on the specialized cells of this system, T lymphocytes, may lead to a better understanding of the mechanisms underlying immune dysfunction. It is proposed that zinc is necessary for the proper functioning of the zinc finger proteins involved in the T lymphocyte signal transduction pathway. An examination of this theory requires a review of T lymphocyte activation and signal transduction.

As discussed earlier, the successful stimulation of T lymphocytes by antigen leads to direct target destruction via cytotoxic T lymphocytes, as well as coordination of the immune response mediated by helper T lymphocytes. Two key signals are necessary for T lymphocyte activation. The first signal, directed by the ligation of the antigen to the T cell receptor (TCR)/CD3 complex, will confer the specificity of the response. The ligation of other T cell surface receptors, such as CD4 and CD8, expressed on T helper and cytotoxic T cells respectively, with their respective major histocompatibility complex markers (which aid in immune cell communication) on the antigen presenting cell (B lymphocyte or macrophage) will transmit the second, co-stimulatory signal. This co-stimulatory signal has been shown to enhance TCR mediated signals (Weil and Veillette, 1996). Together, these interactions create a cascade of biochemical reactions, enabling

signals to be transmitted from the cell surface to the nucleus, resulting in the cellular response.

The exact pathways in T lymphocyte signal transduction are unclear, however a hypothesized sequence of events has been made (Weiss, 1989). A discussion of the early events in the T lymphocyte signal transduction pathway will be emphasized (Figure 2). One of the first biochemical events following the co-stimulation of the TCR and CD4/8 cell surface receptor molecules is an increase in tyrosine phosphorylation of signal transduction proteins, a biochemical process shown to be essential for T lymphocyte activation. In fact, inhibition of protein tyrosine kinase activity has been shown to abolish T lymphocyte activation (June et al, 1990). The association of a tyrosine kinase with TCR and CD4/8 receptors seems likely, as these receptors do not possess catalytic activity, but they are necessary for propagation of the signal. Several lines of evidence have shown that $p56^{lck}$, a tyrosine kinase may have an important role as an early signaling protein in the T lymphocyte signal transduction pathway.

$p56^{lck}$ is a lymphoid specific protein tyrosine kinase (PTK) which is principally expressed in T lymphocytes (Weil and Veillette, 1996), although marginally expressed in B lymphocytes (Gold and Deranco, 1994). $p56^{lck}$ is a member of the src family of PTKs, known to be associated with the cytoplasmic domains of cell surface receptors. Supporting evidence of the association of $p56^{lck}$ and CD4 or CD8 receptors stems from co-immunoprecipitaion assays that demonstrate the presence of an intrinsic association of $p56^{lck}$ with the cytoplasmic domains of CD4 and CD8 (Barber et al, 1989; Veillette et al, 1988).

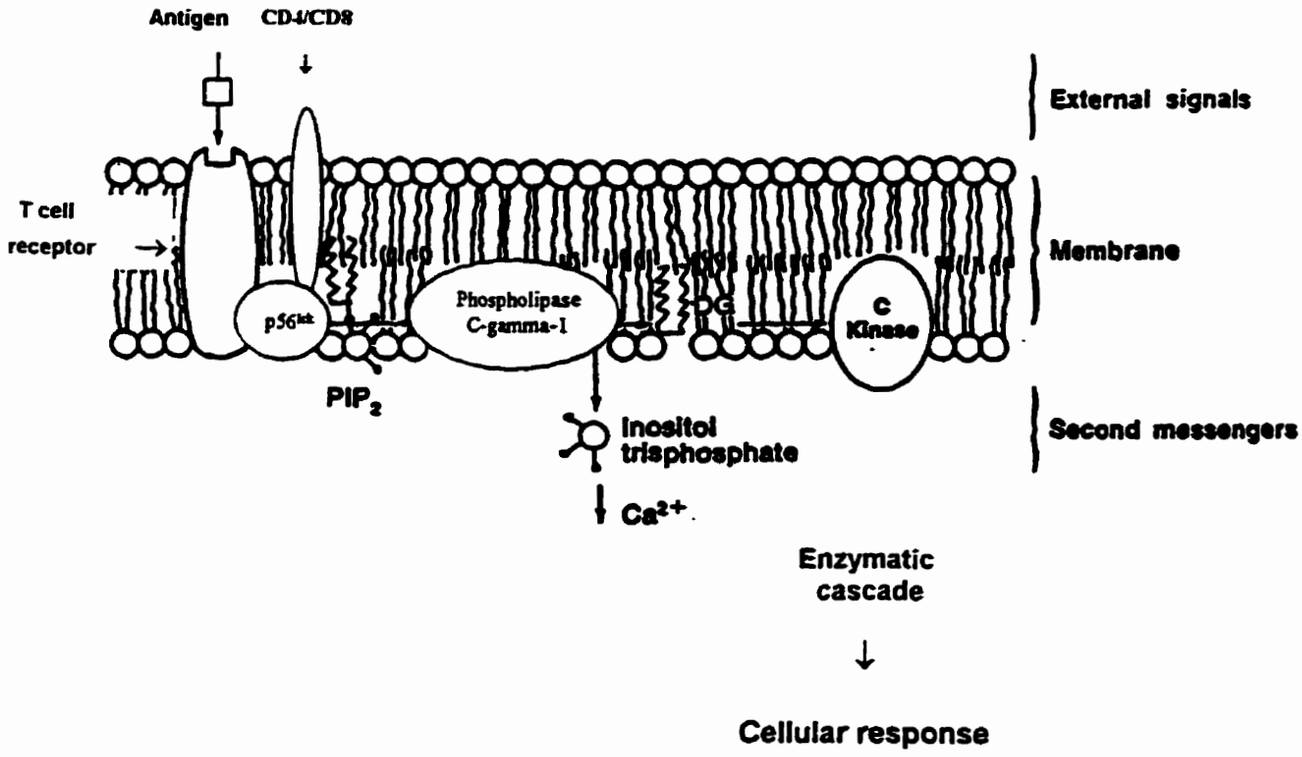

Figure 2: Early events in the T lymphocyte signal transduction pathway
adapted from: Ralws, 1987

Furthermore many researchers have shown that stimulation of co-receptors CD4 or CD8 results in the activation of $p56^{lck}$. It is hypothesized that once activated by CD4 or CD8, $p56^{lck}$ would be able to associate with various surface receptors. For example, co-immunoprecipitation assays have demonstrated that $p56^{lck}$ is associated with the TCR/CD3 molecule following T lymphocyte stimulation. It has been suggested that activated $p56^{lck}$ brought into the vicinity of the TCR/CD3 molecule would augment TCR signals by phosphorylating CD3 (Weil and Veillette, 1996). These results suggest that $p56^{lck}$ is associated with surface receptors and that once activated, this tyrosine kinase may play an important role in propagating the signal.

Activated $p56^{lck}$, phosphorylated on tyrosine residue 394, has been shown to be associated with other signaling molecules in the pathway. Co-immunoprecipitation experiments have proven to be useful in the search for the targets of $p56^{lck}$, as an association between $p56^{lck}$ and phopholipase C gamma-1 (PLCγ1) was found in stimulated T lymphocytes (Weber et al 1992). Supporting evidence of this stems from observations of tyrosine phosphorylation of PLCγ1 following TCR stimulation (Sancho et al 1992), although PLCγ1 is not directly associated with the TCR. PLCγ1 functions as a second messenger by "passing on" the signal via hydrolysis of phophoinositol bisphosphate into diacylglycerol (DG) and inositol triphosphate (IP3). These two metabolites are implicated in signal transduction since DG leads to the activation of PKC and IP3 to an increase in intracellular calcium, biochemical events shown to be necessary in signaling pathways. This provides evidence that activated $p56^{lck}$ can in turn activate other signaling molecules within the pathway, thereby propagating the signal and initiating a cascade of reactions leading to the desired cellular response.

Numerous associations of $p56^{lck}$ with surface receptors as well as intracellular targets have been identified (Anderson et al,1994) and indicate that this tyrosine kinase may play a central role in coordinating early signal transduction events (Figure 3). Investigations as to the nature of these associations has enhanced our current understanding of the functional roles of $p56^{lck}$ and has helped in defining early events in the T lymphocyte signal transduction pathway, such as the interaction of the CD4 surface molecule and $p56^{lck}$.

iii) Interaction of $p56^{lck}$ with CD4 and CD8 is mediated by a zinc finger domain.

Several lines of evidence suggest that the association of $p56^{lck}$ with co-receptor surface molecules CD4 and CD8, is mediated by a zinc finger domain, formed by the association of zinc and cysteine residues. Sequencing and point mutation experiments have shown that the interaction involves the binding of a common cysteine motif in the cytoplasmic tails of CD4 and CD8 molecules to a cysteine motif in the amino terminal sequence of $p56^{lck}$ (Turner, 1990). Point mutations of the cysteine residues involved in the $p56^{lck}$ and CD4 or CD8 interaction prevent their association as demonstrated by co-precipitation experiments (Turner, 1990). In addition, evidence supporting this notion has been provided by Glaichenhaus (1991), whereby point mutants did not respond to T cell stimulation if the cysteine motifs required for the association with $p56^{lck}$ were mutated. These results suggest that $p56^{lck}$ is associated with surface receptors CD4 and CD8 and that their interaction is mediated by cysteine motifs.

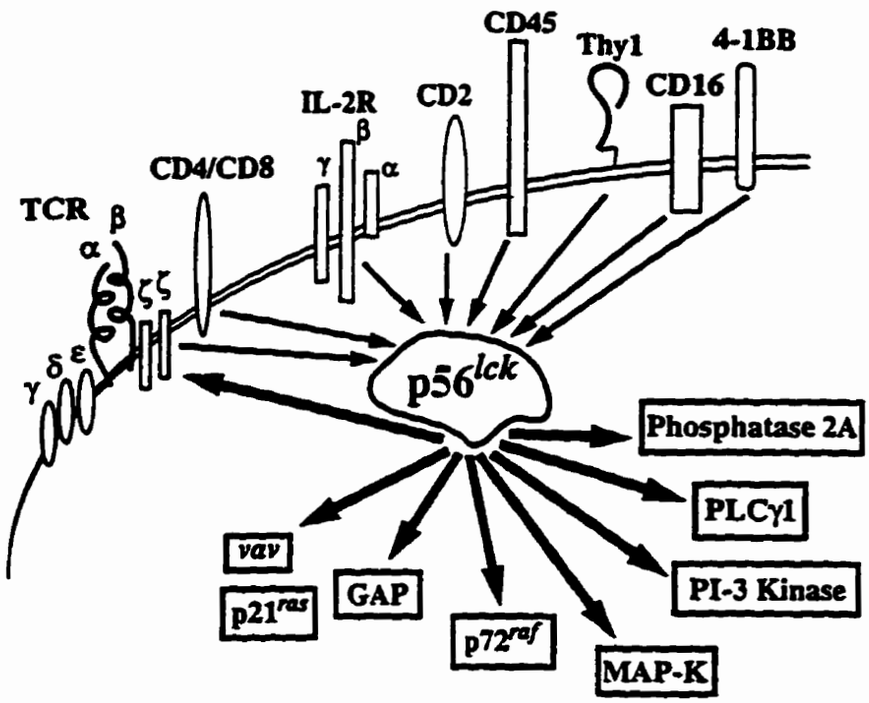

Figure 3: Associations of $p56^{lck}$ with surface receptors and potential targets of phosphorylation

Source: Anderson et al (1994)

The proposal that disulfide bonds were responsible for the interaction of $p56^{lck}$ with CD4 and CD8 was dismissed following experiments involving polyacrylamide gel electrophoresis of $p56^{lck}$ and CD4 or CD8 co-precipitates under reducing conditions (Turner, 1990). Co-precipitates were not separated under these reducing conditions, indicating that their association is not mediated by disulfide bonds (Tuner, 1990). Suggestions of a metal ion stabilizing the interaction have been proposed following the observation that micromolar concentrations of zinc (in the absence of other divalent cations) elicited tyrosine phosphorylation of $p56^{lck}$ (Pernelle et al, 1991). These investigators also studied the effects of various other metal ions on tyrosine phosphorylation of $p56^{lck}$ and found that the effect of other divalent cations, such as cobalt, iron, cadmium and magnesium was not as significant as the effect of zinc.

Further evidence for the involvement of zinc is derived from experiments where zinc chelation disrupts the association of $p56^{lck}$ with CD4 or CD8 as assessed by co-precipitation assays (Turner, 1990). Also, decreased functionality of zinc finger proteins following chelation of zinc was demonstrated in vitro for the transcription factor SP1 (Zeng et al, 1991). In addition, changes in zinc concentration in T lymphocytes after activation, measured by X-ray fluorescence and plasma emission spectroscopy, suggest a role for zinc in T lymphocyte signal transduction pathways (Csermely and Somogyi, 1989).

The evidence provided by in vitro experiments has demonstrated that zinc and its associated cysteine motifs are integral components of zinc finger domains. This has been demonstrated by non-functionality of zinc finger proteins following chelation or point

mutation experiments. To date, information on the effects of dietary manipulation on zinc finger proteins is lacking. It would be of interest to investigate the effects of dietary zinc and sulfur-containing amino acids on the functioning of $p56^{lck}$ since alterations in this early signal transduction protein may have significant downstream effects.

Models of malnutrition and zinc deficiency syndromes

Due to the diversity in the animal models used in defining malnutrition and zinc deficiency syndromes, a discussion of the models described in the literature and in this thesis is warranted.

Currently, discrepancies concerning models of malnutrition exist in the literature. This is due to the use of various approaches for creating experimental models of malnutrition, combined with inconsistent terminology. Protein energy malnutrition (PEM) has been defined as the type of malnutrition produced by a diet deficient in protein and energy (Armour-Forse, 1994). However, the term PEM has been used extensively in the literature to describe a whole spectrum of malnutrition syndromes ranging from protein malnutrition to energy malnutrition.

Pocino et al (1987) fed mice a nutritionally complete diet in restricted amounts and defined the type of malnutrition that was induced as PEM. By restricting food, both protein and energy were thought to be deficient. However, malnutrition resulting from a diet deficient in protein is commonly used to induce PEM, since protein deficient animals have been shown to have reduced food (energy) intake. However, reduced food intake in protein deficient animals is dependent on the extent of dietary protein deficiency since

Kirsh et al (1968) observed a reduction in food intake in rats receiving a 5% protein diet over a period of 9 weeks, whereas 8 % and 12 % protein diets did not adversely affect food intake in young rats. Furthermore, it is possible that weanling animals may be more susceptible to dietary protein deficiency induced anorexia. In the current study, adult mice were fed a 2% protein diet, but it is not known whether these mice had a decrease in food intake, due to difficulties in recording food intake when mice are fed powdered diets.

In the current study, the type of malnutrition was determined biochemically and termed accordingly (protein or energy malnutrition). Further biochemical analysis, such as carcass lipid and protein (Woodward and Lee, 1996) may be required in order to identify protein-energy malnutrition (characterized by a decrease in overall lipid stores and visceral and somatic protein status; Zeman, 1991).

Previously, many experimental models were designed to assess the effects of malnutrition and zinc deficiency syndromes on growth by using weanling or young animals. Assessment of the effects of nutrient and energy deprivation on parameters of zinc and immune status in young animals may be confounded by growth since this is a period of high nutrient and energy requirements. Therefore, an animal model comprised of immunologically mature young adult mice (4 months) was designed to provide a basic model of the effects of the dietary treatment on immune function, uncomplicated by rapid growth and development of the immune system.

Furthermore, models of zinc or malnutrition vary with respect to level of dietary deficiency and length of experiment. Pocino and Malave (1984) have investigated chronic protein deprivation (4 % and 8%) over a long period of time (12 weeks), whereas Woods and Woodward (1991) have implemented a more severe form of dietary protein

restriction (0.6%) over a shorter period of time (2 weeks), in young mice. Studies assessing the severity of zinc deprivation have also been conducted by feeding diets moderately (6 ppm; Zhou et al, 1993) or severely deficient in zinc (0.9 ppm; Cook-Mills and Fraker).

Other than the level of nutrient deficiency, few variations exist in experimental models of dietary zinc deficiency. There are two approaches used to control for zinc deficiency related anorexia; pair-feeding and paired-weight. A pair-fed group is fed amounts of diet (zinc adequate) equal to that consumed by the zinc deficient animals. Typically, pair-fed animals have increased body weight compared to zinc deficient animals due to their increased feed efficiency. Cook-Mills and Fraker (1993) reported that mice receiving zinc deficient diets weighed significantly less (19%↓) than their pair-fed counterparts. Alternatively, a paired-weight group is fed restricted amounts of a zinc adequate diet in order to achieve the same weight as in the dietary zinc deficient group.

In the present study, not only was it of interest to study zinc deficiency, but also malnutrition syndromes. By inclusion of an energy restricted group, zinc deficiency related anorexia could be controlled for and at the same time provide a model for energy-type malnutrition. Contrary to the above mentioned pair-feeding, food intake was restricted in amounts (ER group) in order to maintain the same body weight as the ZnDF group. This approach provided an opportunity to control for the effect of weight loss, rather than reduced food intake on the various parameters of interest.

An important factor which is missing in experimental animal models of zinc deficiency is concomitant protein deficiency, since in a clinical setting zinc deficiency is commonly associated with protein deficiency. Thus, a model of combined dietary zinc

and protein deficiency was included in this study. Not only would such a model permit the investigation of dietary effects of these nutrients on zinc finger proteins, but also it would better represent the type of zinc deficiency commonly observed in humans.

A model with moderately severe nutrient deficiencies (1 ppm Zn and 2% of total calories from protein) for a short time period (4 weeks) was used as part of our model since such a restriction would provide significant differences if dietary factors had significant effects.

II. Study rationale

In order to better understand the mechanisms underlying zinc deficiency pathogenesis, particularly immune dysfunction, it is necessary to tackle the problem holistically. This can be accomplished by understanding the relationships between nutritional zinc deprivation and developments occurring at the molecular, biochemical, cellular, organ and whole body levels. Also, identifying changes in these indices and comparing them to other malnutrition syndromes will help to characterize the type of malnutrition associated with zinc deficiency.

In addition, it would be of interest to recognize any immunological alterations induced by dietary zinc deficiency, in comparison to various types of malnutrition. Since zinc deficiency is commonly associated with protein or energy type malnutrition, investigations of the effects of zinc deficiency occurring concomittantly with protein or energy malnutrition will help to define their interaction.

Zinc is thought to play an important role in maintaining structural integrity and functionality of zinc finger proteins, many of which are found within the T lymphocyte signal transduction pathway. It is hypothesized that dietary deficiencies of zinc, and sulfur containing amino acids (cysteine) may adversely affect zinc finger protein structure and function as is demonstrated in vitro.

The objective of this thesis is to investigate the effects of dietary zinc deficiency and malnutrition syndromes [protein deficiency alone or combined with zinc deficiency, and energy restriction] compared to a nutritionally adequate control group on the expression of $p56^{lck}$, an early T lymphocyte signal transduction zinc finger protein, zinc

status (serum and femur zinc), lymphoid organ assessment (spleen weight and total cell population) and type of malnutrition (serum albumin and liver lipid concentrations).

In order to meet these objectives, models of dietary zinc deficiency, protein deficiency, combined zinc deficiency and protein deficiency, and energy restriction were developed in immunologically mature young adult mice. Furthermore, a method for T lymphocyte isolation from murine spleen for subsequent Western immunoblotting analysis was developed, since prior immunoblotting analyses of T lymphocyte signal transduction proteins (ie. $p56^{lck}$) have been conducted using cell lines.

Although previous studies have investigated the effects of zinc deficiency or malnutrition on immunity by performing tests of immune function, such tests do not provide information of the molecular mechanisms underlying immune dysfunction. Research at the molecular level can now be accomplished due to significant advances in molecular techniques (Western Immunoblotting). Furthermore, the development of biological reagents such as antibodies for use in T lymphocyte immuno-affinity columns makes it possible to isolate viable T lymphocytes from mice. These technical advances permit the investigation of the effects of nutritional deficiencies at the molecular level in T lymphocytes. Recent characterization of the T lymphocyte signal transduction pathway, particularly signal transduction proteins such as $p56^{lck}$, has provided knowledge which can be used to investigate the molecular mechanisms underlying the immune dysfunction observed in zinc deficiency and malnutrition syndromes.

III. Materials and Methods

Animals and Diets

Young adult (2 month old) female mice of the medium responder strain C57BL/6 (Charles River Laboratories, St.Constant, PQ) were acclimatized to nutritionally complete standard chow. Mice were maintained in an environment of controlled temperature (21-23^{0}C), humidity (55%) and light cycle (14 hours light/ 10 hours dark). At the immunologically mature age of 4 months old, 5 mice were randomly selected and terminated to determine baseline parameters.

The remaining 45 mice were randomly assigned to one of the five dietary treatment groups: ZnDF + LP (< 1 ppm Zn + 2 % protein), ZnDF (<1 ppm Zn, 15 % protein), LP (2% protein, 30 ppm Zn), or the nutritionally complete control diet, C (30 ppm Zn, 15 % protein). The energy restricted group (ER) was fed the control diet in amounts necessary to follow weight loss trends or to maintain body weight as observed in the ZnDF group (due to a decrease in appetite secondary to zinc deficiency). Therefore, not only will the ER group provide information on alterations in biochemical parameters accompanying energy restriction, but also will serve as a control group for the ZnDF group to interpret changes due to zinc deficiency *per se* or weight loss.

Diets based on the AIN-93M formulation (Reeves, 1993) were fed *ad libitum*, except for the ER group, and distilled water was provided. Diet ingredients were purchased from Harland Teklad (Madison, WI), with the exception of the soy oil (Vita Health, Winnipeg, MB). The diet formulation is provided in Table 2. Verification of the concentration of zinc contained in the diets was performed as described later under zinc analysis.

TABLE 2

Diet Composition[1]
(g/kg)

Ingredient	ZnDF + LP	ZnDF	LP	C
Egg White	25	150	25	150
Soybean oil	40	40	40	40
Mineral mix (zinc free)[2]	35	35	35	35
Zinc premix[3]	-	-	40	40
Vitamin mix[4]	10	10	10	10
Choline	2.5	2.5	2.5	2.5
Dextrose	887.5	762.5	887.5	752.5

[1] Diet ingredients were purchased from Harlan Teklad (Madison, WI) with the exception of the soybean oil (Vita Health, Winnipeg, Manitoba).
[2] AIN-93 Zinc free mineral mix (Harlan Teklad).
[3] Zinc premix (11.55 g zinc carbonate/1000 g cerulose)
[4] AIN-93 vitamin mix (Harlan Teklad)

Care was taken to avoid zinc recycling and contamination by housing animals in stainless steel hanging cages with mesh bottoms, and providing distilled water in plastic bottles with stainless steel sipper tubes. In addition, the zinc deficient groups were placed on the upper rows of the cage rack.

Mice received their respective dietary treatment for a duration of 4 weeks. Body weights were determined weekly, with the exception of the daily weighing of the energy restricted group. Animal care was provided in accordance with the protocol approved by the Local Animal Care Committee (University of Manitoba).

Tissue Collection

At the end of the 4 week feeding trial, mice were terminated by $C0_2$ asphyxiation and cervical dislocation according to the Canadian Council on Animal Care Guidelines. Animals were weighed, followed by collection of trunk blood which was placed immediately on ice prior to centrifugation (1290 x g for 15 minutes, Beckman Model TJ-6 centrifuge) to obtain serum. The spleens were removed aseptically, weighed and then processed immediately. Femurs with their accompanying musculature, and livers were dissected. Liver weights were recorded. Tissues were frozen in liquid nitrogen and stored at -80^{0}C.

Optimized T lymphocyte lysate preparation

i) Mononuclear cell suspension and count

All reagents (molecular biology grade) were purchased from Sigma Chemical Company (St-Louis, MO) with the exception of acid solutions, solvents and standard laboratory materials (VWR Canlab, Mississauga, ON) unless otherwise indicated. A spleen cell suspension was prepared in a round petri dish under aseptic conditions in a

Nuaire biological tissue culture hood (Plymouth, MN). Sterile bent needles were used to scrape spleen cells into 5 mls of sterile PBS pH 7.3 (80.0 g/l NaCl, 2.0 g/l KCl, 11.5 g/l $Na2HPO_4$ x $7H_20$, 2.0 g/l KH_2P0_4) supplemented with 2% fetal calf serum (FCS), (Gibco, Grand Island, NY) creating a total spleen suspension containing mononuclear and polyphomornuclear cells, erythrocytes and dead cells. The cell suspension was transferred to a 15 ml conical Falcon tube. Cells remaining in the petri dish were recovered by a brief rinse with 2 mls PBS/2% FCS. The suspension was centrifuged for 5 minutes at 300 x *g*. The supernatant was discarded and the cells were resuspended and gently inverted for 2 minutes in 2 mls of Tris-buffered ammonium [working solution 90 mls 0.16M NH_4Cl, 10 mls 0.17M Tris pH 7.65; adjusted to pH 7.2] in order to lyse erythrocytes. The lysis solution was diluted by adding 3 mls of PBS/2% FCS and then the cell suspension was centrifuged. This procedure was repeated once more to ensure that erythrocytes were no longer evident in the pellet. Cells were washed twice in 8 mls of PBS/2% FCS, then resuspended in 3.5 mls of PBS/2% FCS, a volume appropriate for cell counting.

The total nucleated spleen cell count was determined using a AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY) following the directions provided for the counting of white cells. Ten ul of the cell suspension was diluted with 990 ul of 5% acetic acid in order to lyse any residual red blood cells (dilution factor = 100). Then 10 ul was loaded onto each counting chamber.

Calculation:

$$\frac{\text{Average cell count x dilution factor (100) x 10 (0.1 mm depth)}}{\text{4 (number of square mm counted)}} \times 3.5\text{ml} = \text{cells/3.5ml}$$

ii) T lymphocyte isolation and count

Mouse T immunocolumn (Biotex Laboratories, Edmonton, AB) were set up and run according to the protocol provided by the manufacturer. Column set up and activation was completed one hour prior to animal terminations. After setting the column on a support, the column was washed with 20 mls of PBS/2% fetal calf serum at a rate of 6-8 drops/minute. The column reagent, containing antibodies provided by the manufacturer, was reconstituted with 1.5 mls of PBS/2% fetal calf serum, then allowed to incubate on the column for approximately 2 hours following the wash. Excess column reagent was then washed off with 15 mls of PBS/2% fetal calf serum.

Splenocyte suspensions from individual mice were applied to separate columns. Cells (1×10^8), adjusted to a concentration of 5×10^7 cells/ml were allowed to flow through the column at a rate of 6-8 drops/minute, and 20 mls of the eluant was collected in two, 15ml conical Falcon tubes. Cells were centrifuged at 300 x *g* for 10 minutes and then resuspended in 0.8ml and counted as described above. The Trypan blue exclusion test was performed to verify T lymphocyte viability. A suspension of Trypan blue 0.4 %, PBS and a 800 ul sample in proportions of 2.5, 1.5 and 1 respectively, were counted as described above. Non-viable cells are dyed blue, as their ruptured membranes are not able to exclude the dye. Both viable (clear) and non-viable cells were counted. T lymphocytes were then re-pelleted in preparation for cellular lysis.

iii) T lymphocyte lysis

The T lymphocyte pellet was lysed by resuspension in 160 ul of ice-cold RIPA buffer [1% Nonidet P-40 (BDH Laboratory Supplies, Poole, England), 0.25% deoxycholate, 150mM NaCl, 50mM Tris pH 7.5) containing protease inhibitors (1mM

sodium orthovanadate, 1mM EGTA, 0.5% Aprotinin, 12.5 ug/ml Leupeptin, 1mM Phenylmethylsulfonylfluoride], for 30 minutes (Kanner et al, 1990). The lysates were passed through a 27 gauge needle and then centrifuged for 10 minutes at 15600 x g (Eppendorf Centrifuge # 5414) to pellet the nuclear fraction. The supernatant containing the cytosolic fraction was moved to fresh tubes and frozen at -80^{0}C until the measurement of protein concentration.

iv) Protein Assay

A protein concentration of 1 ug/ul was needed for SDS-PAGE. The BCA Protein Assay method (Pierce, Rockford, IL), based on the biuret reaction (Lowry, 1951), was used. A set of protein standards (0.1 - 1.0 mg/ml) were prepared from bovine serum albumin (2mg/ml) provided by the assay kit, using RIPA buffer as the diluent. The standard curve was verified for linearity. Fifty ul of each protein standard and sample (20 ul diluted to 50ul with RIPA, dilution factor = 2.5), were mixed by vortexing with 1 ml of the working reagent, then incubated for 30 minutes at 37^{0}C. Tubes were vortexed, and contents transferred to polystyrene cuvettes then read at 562 nm on a Milton Roy Spectronic 3000 Spectrophotometer (Fisher Scientific, Nepean, ON). Samples were brought to a concentration of 1ug/ul with RIPA buffer.

Calculation: $\frac{\text{Absorbance of sample}^* - \text{Absorbance of blank}}{\text{Absorbance of standard - Absorbance of blank}} \times \text{Conc. of Standard}$

*The average of duplicate samples was used.

Western Immunoblotting

This procedure was used to identify the T lymphocyte signal transduction protein, $p56^{lck}$ N terminal domain (NT) using a polyclonal antibody, Anti-Human Lck Kinase NT, rabbit antiserum (Upstate Biotechnology Incorporated, Lake Placid, NY). Apparatus and reagents were purchased from Bio-Rad Canada (Mississauga, ON) unless otherwise indicated. Buffer formulations for SDS-PAGE and Western Immunoblotting were provided by Bio-Rad, and Boerhinger Mannheim (Laval, PQ, Canada). Five ug of the protein sample (5 ul) was solubilized by boiling for 4 minutes with equal volume of 2 x SDS sample buffer [4% SDS, 20% glycerol w/v (Sigma Chemical Co., St-Louis, MO), 10% betamercaptoethanol (BDH Laboratory Supplies, Poole, England) 0.05% bromophenol blue, 0.5M Tris ph 6.8). Proteins contained within the sample were then separated according to molecular weight by SDS-PAGE, using a 10% SDS separating gel [5 ml H_20, 1.7 ml 30% Acrylamide mix, 1.3 ml 1.5M Tris pH 8.8, 0.05 ml 10% sodium dodecyl sulfate (SDS), 0.05ml Ammonium persulfate (APS) made fresh daily, 0,002 ml TEMED] and a 5 % stacking gel [1.4 ml H_20, 0.33 ml 30% acrylamide mix, 0.25 ml 1.0 M Tris pH 6.8, 0.02 ml SDS, 0.02 ml APS, 0.002 ml TEMED]. Electrophoresis took place using 500 mls electrode buffer pH 8.3 (3 g/L Tris base, 14.4 g/l Glycine, 1g/l SDS) at 170 V (Power Pac 200) for 1 hour in the Mini-Protean II electrophoresis cell.

Separated proteins were then transferred, electrophoretically (Mini Trans-Blot Electrophoretic Transfer Cell), to a nitrocellulose membrane (0.2 micron) using transfer buffer pH 8.3 (3.03g/l Tris, 14.4 g/l glycine, 200 ml/l methanol) and the transfer cell at 100 V for 1 hour. The membrane was then equilibrated in Tris buffered saline pH 7.5 (6.05 g /l Tris, 8.76 g/L NaCl, pH adjusted with HCl). The reagents used in the following

description of $p56^{lck}$ (NT) immunodetection were purchased as a kit (BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) from Boehringer Mannheim, unless otherwise indicated.

Immunodetection of specific antigens, particularly $p56^{lck}$, is possible since separated proteins have been blotted onto the surface of the nitrocellulose membrane. To avoid non-specific binding, the remaining binding sites were blocked with a 1% blocking solution on an orbital shaker for 1 hour at room temperature. Probing for $p56^{lck}$ NT was accomplished by using a 1:1000 dilution of Anti-Human lck kinase NT in 0.5% blocking reagent, shaking at 4^0C overnight. The membrane was washed twice with TBS-T [1 ml Tween 20/ 1 L TBS (50 mM Tris base, 150 mM Sodium Chloride, adjusted to pH 7.5 with HCl] for 10 minutes each, then equilibrated with 0.5% blocking solution, likewise for 10 minutes each. The antigen-antibody complex was then identified with a anti-mouse/rabbit IgG horseradish peroxidase (POD) employing a 1:2500 dilution in 0.5% blocking solution. Following a 2 x 15 minute wash in TBS-T, this enzyme coupled secondary antibody is allowed to react for 1 minute, with the luminescent substrate contained in the detection solution in order to visualize the protein bands by photographic exposure. In order to analyze the expression of $p56^{lck}$, bands were scanned by image analysis and assigned arbitrary units which represent band area combined with grey intensity (see statistical analysis). These data were then normalized by log transformation and analyzed statistically. Significant differences between means were determined using Duncan's multiple range test.

India Ink Staining

The separated proteins blotted onto the membrane can be stained with India Ink for visualization of transfer efficiency and band identification. Membranes were washed in Tween 20 solution (0.3% v/v Tween-20 in PBS) at 37^{0}C 3 times for 30 minutes each on an orbital shaker, followed by two 30 minute washes at room temperature. The membrane was allowed to stain in India Ink solution [0.1% India Ink (Osmiroid International, Hampshire, England) in Tween 20 solution] on an orbital shaker for 3 hours at room temperature. The membrane was then washed in Tween 20 solution until grey bands appear on a white background and then air dried.

Analysis of Zinc status

Femurs and serum were assessed for zinc using Atomic Absorption Spectrophotometry. Frozen femurs were thawed and scraped of all musculature and connective tissue with a scalpel blade. Wet weights were taken prior to drying for 48 hours at 85^{0}C. Dry weights were then obtained preceding wet-ashing, a technique used to digest tissues (Clegg et al, 1981). Each femur was placed in a dry, acid washed test tube containing 1 ml of concentrated nitric acid. Femurs were allowed to digest for 24 hours at room temperature, followed by heating for 48 hours at 85^{0}C. Digests were diluted to 10 ml with distilled, deionized water, followed by analysis by atomic absorption spectroscopy, using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, ON).

Diets were analyzed for zinc content by digesting 2 gm of the zinc deficient diets and 1 gm of the zinc adequate diets in concentrated nitric acid for 24 hours, followed by

48 hours of heat treatment at 85°C. Samples were diluted to 10 mls with distilled water. Zinc standards (0.1-1ppm) were prepared from zinc atomic absorption standard (1000 ppm, # H595-01 Mallinckrodt, Paris, Kentucky), and quality control was monitored using bovine liver standard reference material 1577b (U.S. Department of Commerce, National Insititute of Standards and Technology, Gaithersburg, MD).

Femur zinc concentration calculation:

$$\frac{\text{Sample Zinc concentration x dilution factor (10)}}{\text{femur dry weight}} = \text{Femur Zinc ug/g dry weight}$$

Serum zinc was assessed directly by a 10 to 20 fold dilution of the sample (approximately 100 ul) to provide enough sample for aspiration through the nebulizer of the atomic absorption spectrophotometer.

Serum zinc concentration calculation:

Sample Zinc concentration x dilution factor = Serum Zinc ug/ml

Analysis of protein status

i) Serum Albumin

Serum albumin concentration was determined using Sigma Diagnostics Albumin Reagent containing bromcresol green (BCG). The method is based on the procedure outlined by Doumas (1971). BCG is bound to albumin, producing a blue green color. Standards (0.5-5 g/dl) were prepared from human serum albumin (Sigma), and the standard curve was verified for linearity, using water to set the absorbance reading to zero (wavelength = 628 nm). One ml of Albumin Reagent was aliquoted into each tube,

followed by the addition of 10 ul of the standard or sample at timed intervals. Samples were mixed by gentle inversion, and read at 1 minute or less following mixing.

Serum albumin concentration calculation:

$$\frac{\text{Absorbance sample - Absorbance blank}}{\text{Absorbance standard - Absorbance blank}} \times \text{Conc. of standard} = (\text{g/dL})^{*}$$

*To convert to SI units multiply by 144.9 = umol/L

ii) Liver lipid

Protein malnutrition has been associated with an accumulation of liver lipid (Sato et al, 1996). The determination of liver lipid was by the Folch (1956) method, where specific ratios of chloroform, methanol and water are used to extract the lipid fraction. Each liver was thawed, weighed and homogenized in 22 mls of a 2:1 mixture of chloroform and methanol, respectively, for 30 seconds (setting 4) using a Polytron homogenizer (model PT 1020 3500, 115 volt, Brinkmann Instruments, Rexdale, ON). The homogenate was passed through a #1 Whatman filter and the volume of the eluate, collected in a 25 ml graduated cylinder was recorded. Twenty percent of this volume was added as water and then shaken. The milky suspension was covered with a stopper and allowed to separate overnight.

The volume of the lower chloroform layer containing lipid was recorded, and this was followed by removal of the upper methanol layer. Ten mls of the chloroform layer was placed in dried and weighed 25 ml glass vials, and the chloroform was evaporated in a heated water bath (OA-SYS heating system, Organomation Associates, Berlin, MA) with nitrogen air (0.7 Kg/cm^2) for 1 hour. The vials containing lipid were allowed to cool in a dessicator, and then weighed to determine the lipid content.

Calculation:

$$\frac{\text{vial dry weight containing lipid - vial dry weight}}{\text{volume of chloroform used (10 ml)}} \times \text{volume of the actual chloroform layer} = \text{g lipid/g of tissue used}$$

Statistical analysis

Differences between dietary treatment groups were analyzed by ANOVA (SAS software release 6.04, SAS institute, Cary, NC) and significant differences between means were determined using Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

The data obtained from image analysis (expression of $p56^{lck}$) were not normally distributed, therefore these data sets were normalized by log transformation prior to analysis by ANOVA. Correlation analysis (zinc status vs expression of $p56^{lck}$) was performed using Pearson's correlation coefficient.

IV. Results

Assessment of zinc status and malnutrition type

Common clinical signs of zinc deficiency and protein malnutrition such as hair loss and dermal lesions were observed in some animals in the ZnDF and LP groups, whereas mice from the combined ZnDF + LP group were the most severely affected. Growth retardation, a general indicator of malnutrition and Zn deficiency was observed in all groups compared to the control mice. Anorexia is a common clinical sign of Zn deficiency and protein malnutrition, however assessment of food intake was not accomplished due to difficulties in obtaining accurate feed intake data from mice fed purified powdered diets.

The effects of dietary treatment, namely ZnDF + LP, ZnDF, ER, LP or C on body weight, liver weight, and zinc and protein status are presented as means and as percentage change from the C group (Table 3). Individual standard error of the mean for all parameters that were assessed is presented in appendix A. Two mice from the ZnDF+LP group and one mouse from the LP group were severely affected by dietary treatment, and therefore were terminated prior to the end of the experiment. At baseline, the mean body weight was 22.2 g. After a four week feeding trial, mice fed the C diet experienced a mean weight gain of 2.6 g. Dietary deficiencies of zinc, protein or energy did not result in any weight gain or loss when compared to baseline mice, however, mice fed deficient diets weighed significantly less (approximately 15%) than the C group.

Zinc status was evaluated by analyzing serum and femur zinc concentrations which represent short term and long term zinc body pools, respectively. In comparison to the

C group, serum zinc levels were significantly lower (approximately 50%) in mice receiving Zn deficient diets, however no significant differences were observed between the ZnDF + LP and ZnDF groups (0.59 and 0.64 μg/ml, respectively). Although serum zinc concentrations in the ER group were not significantly different from the C group, there was a small, yet significant decrease in serum zinc concentration in the LP compared with the C group (1.01 vs 1.23 μg/ml, respectively). Similarly, femur zinc concentrations were significantly reduced in the zinc deficient groups compared with the C group; however, the ZnDF group had a significantly lower femur zinc concentration compared with the ZnDF + LP and Baseline groups (174.5 vs 189, 194 μg/g dry weight, respectively). Significant decreases, though not as large as those seen in mice fed Zn deficient diets, were observed in the ER and LP groups compared to the C group (203.4, 206.7 vs 221.1 μg/g dry weight, respectively). The observed decreases in zinc concentration were greater in the serum compared to the femur. This is reflective of serum zinc being depleted more rapidly than femur zinc. It appears that induction of Zn deficiency has occurred in the animals receiving zinc deficient diets as evidenced by significant decreases in these two body Zn pools.

Total liver lipid concentrations can be used in the assessment of the type of malnutrition, since protein-type malnutrition is characterized by an accumulation of lipid in the liver. The LP and ZnDF + LP groups exhibited a significantly higher total liver lipid concentration in comparison to the C group. Also, the ER and ZnDF groups were not significantly different from the C group. These results indicate the presence of fatty livers in mice receiving low protein diets. Mice fed low protein diets also had decreased liver weights in comparison to the C group. Liver weight reductions of 27% and 30% were

observed in the protein deficient groups, LP and ZnDF + LP respectively, whereas the protein adequate diets, ER and ZnDF were not found to be significantly different from the C group. A similar trend was observed when liver weight was expressed as a percentage of body weight.

Serum albumin is commonly used in the assessment of visceral protein status, therefore it can assist in identifying protein malnutrition. Serum albumin was reduced by 2.7% and 4.5% in the LP and ZnDF + LP groups respectively, compared to the C group, although these differences were not significant. Elevated serum albumin was present in mice fed deficient diets adequate in protein, that is the ER and ZnDF groups, compared to the C group. These results demonstrate that protein malnutrition was induced in animals receiving low protein diets, namely the ZnDF+LP and LP groups, and that this type of malnutrition was different from that observed in the ZnDF and ER groups.

Assessment of immunological parameters.

Spleen weight and total spleen cell (splenocyte) counts were assessed since a depression of these parameters is commonly observed in immunodeficiency states. The results for spleen weight, also reported as a percentage of body weight, demonstrate that mice fed zinc deficient diets display a significant decrease in spleen weight (Table 4). Splenocyte counts were significantly reduced in the ER group compared with the ZnDF and ZnDF+LP groups (Table 4). Spleen weight was significantly lower in the ZnDF + LP (↓28 %) and ZnDF (↓25 %) groups, compared to the C group. However, the ER group, which can be interpreted as a food restricted control group for the ZnDF group, experienced a greater reduction in spleen weight compared to the ZnDF group. Also,

significant decreases (40%) were observed in the LP group compared to the C group, although significant decreases were not observed between the LP and ZnDF + LP groups. However, when spleen weight is expressed as a percentage of body weight, spleen weight in the LP group was significantly decreased compared to the ZnDF + LP group.

Total splenocyte counts per spleen were depressed in all dietary treatment groups compared to the C group (Table 4). A depression in splenocyte concentration in the spleen was observed in all dietary treatment groups compared to the baseline group (Figure 5). The largest reduction in splenocyte concentration was observed in the ER group compared to the C group, although the ER group was not significantly different from the ZnDF or LP groups. The number of splenocytes/mg spleen was not different among the ZnDF + LP, LP, and C groups. Although the ER and LP groups had the smallest spleens (by weight, Table 4), the lowest splenocyte number/mg spleen was observed in the ZnDF and ER groups compared to the C group (Figure 5). The effect of the LP diet alone appeared to have a greater effect on spleen weight and splenocyte count (Table 4) than the combined ZnDF+LP diet.

Assessment of Zinc-finger T-lymphocyte protein, $p56^{lck}$

Analysis of $p56^{lck}$ (NT) by Western Immunoblotting was performed in order to evaluate the expression of $p56^{lck}$ (NT) in T lymphocytes from mice fed the various dietary treatments. Subsequent staining of the membrane (containing the molecular weight markers) with India ink serves to identify the bands representing the expression of $p56^{lck}$ (NT) in comparison to other proteins in the T-lymphocyte cell lysate (Figure 6a). Expression of $p56^{lck}$ (NT) was increased in all dietary treatment groups compared to the

C group (Figure 6b). The largest expression of $p56^{lck}$ (NT) was detected in both types of dietary Zn deficiency in comparison to the C group (Figure 6c). The ZnDF + LP had a higher expression, though not significantly different than the ZnDF group. The ER group had a lower expression of $p56^{lck}$(NT) than the ZnDF group and a higher expression than the LP group, however these differences were not significantly different from either group. Although elevated expression was also observed in the LP group, this was not found to be significantly different from the C or ER groups (Figure 6c).

In view of the finding of increased expression of $p56^{lck}$ in the dietary zinc deficient groups, correlation analysis between zinc status and expression of $p56^{lck}$ was conducted (Figure 7). Correlation analysis using Pearson's correlation coefficient (r) revealed significant negative correlations between the expression of $p56^{lck}$ and serum zinc ($r = -0.71$, $p = 0.0002$) or femur zinc ($r = -0.70$, $p = 0.0002$) concentrations, as shown in Figure 7a and 7b, respectively.

Table 3

Effects of dietary treatments on body and liver weights, and various biochemical parameters of zinc and protein status in mice[1]

	Treatment group[2]						
	B	ZnDF + LP	ZnDF	ER	LP	C	Pooled SEM
Body weight *(g)*	22.2^{B}	21.2^{B} 14.5%↓	21.6^{B} 12.9%↓	21.2^{B} 14.5%↓	20.5^{B} 17.3%↓	24.8^{A}	0.51
Serum Zn *(µg/ml)*	1.03^{B}	0.59^{C} 50%↓	0.64^{C} 48%↓	$1.12^{A,B}$ 9%↓	1.01^{B} 18%↓	1.23^{A}	0.05
Femur Zn *(µg/g dry wt)*	194^{C}	189^{C} 14.8%↓	175^{D} 21.1%↓	203^{B} 8%↓	207^{B} 6.5%↓	221^{A}	2.83
Lipid conc. *(mg/g* liver)	38.1^{C}	74.1^{A} 31.6↑	53.1^{B} 5.7%↓	60^{B} 6.6%↑	72.9^{A} 29%↑	56.3^{B}	2.06
Liver weight *(g)*	1.28^{A}	0.90^{C} 29.7%↓	1.10^{B} 14.1%↓	$1.17^{A,B}$ 8.6%↓	0.93^{C} 27.3%↓	1.28^{A}	0.05
% Liver/Body weight	5.76^{A}	4.24^{C} 17.9%↓	5.09^{B} 1.4%↓	$5.51^{A,B}$ 6.8%↑	4.52^{C} 12.4%↓	5.16^{B}	0.16
Serum albumin *(µmol/l)*	568^{A}	430^{C} 4.5%↓	501^{B} 11.3%↑	576^{A} 28%↑	438^{C} 2.7%↓	450^{C}	14.01

[1]Values are means and percent change from controls, n = 9 except for B, ZnDF + LP and LP where n = 5, n = 7 and n = 8, respectively. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] B = Baseline, ZnDF+LP = Zn deficient + 2% Protein, ZnDF = Zn deficient, ER = Energy Restriction, LP = 2% Protein, C = Control.

Table 4

Effect of dietary treatment on spleen weight and total splenocyte count[1]

	Treatment group[2]						
	B	ZnDF+LP	ZnDF	ER	LP	C	Pooled SEM
Spleen *mg*	89.1^{A}	$72.9^{C,B}$ 27.9%↓	75.8^{B} 25.0%↓	58.5^{D} 42.1%↓	$60.9^{C,D}$ 39.8%↓	101.1^{A}	3.90
% Spleen/body weight	$0.40^{A,B}$	0.35^{B} 14.6%↓	0.35^{B} 14.6%↓	0.28^{C} 31.7%↓	0.29^{C} 29.3%↓	0.41^{A}	0.02
Splenocytes per spleen, x 10^{8}	2.44^{A}	1.40^{C} 27.5%↓	$1.12^{C,D}$ 42.0%↓	0.84^{E} 56.5%↓	$1.04^{D,E}$ 46.1%↓	1.93^{B}	0.11

[1]Values are means and percent decrease from the C group, n=9, except for the B, ZnDF+LP and LP groups where n=5, n=7 and n=8 respectively. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2]B=Baseline, ZnDF+LP = zinc deficient + 2% Protein, ZnDF = Zn deficient, ER = Energy Restriction, LP = 2% protein, C = Control.

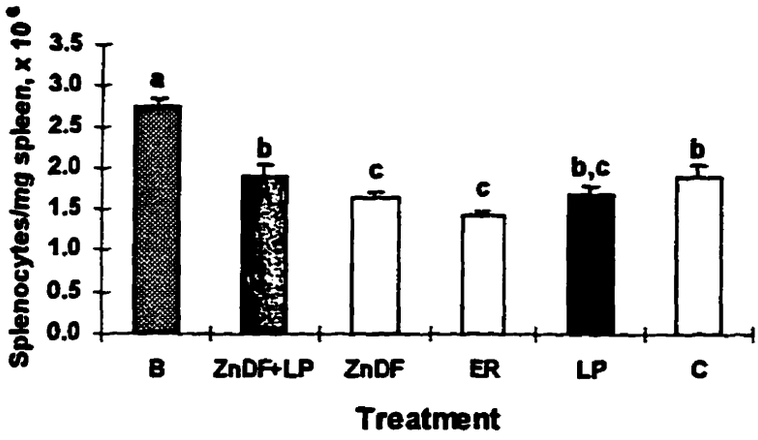

Figure 5. The effect of dietary treatment on splenocytes/mg spleen from mice fed zinc deficient and 2% protein (ZnDF+LP), zinc deficient (ZnDF), energy restricted (ER) or control (C) diets. Columns represent mean +/- SEM, n=9 except for the B, ZnDF+LP and LP groups where n=5, n=7 and n=8 respectively. Lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

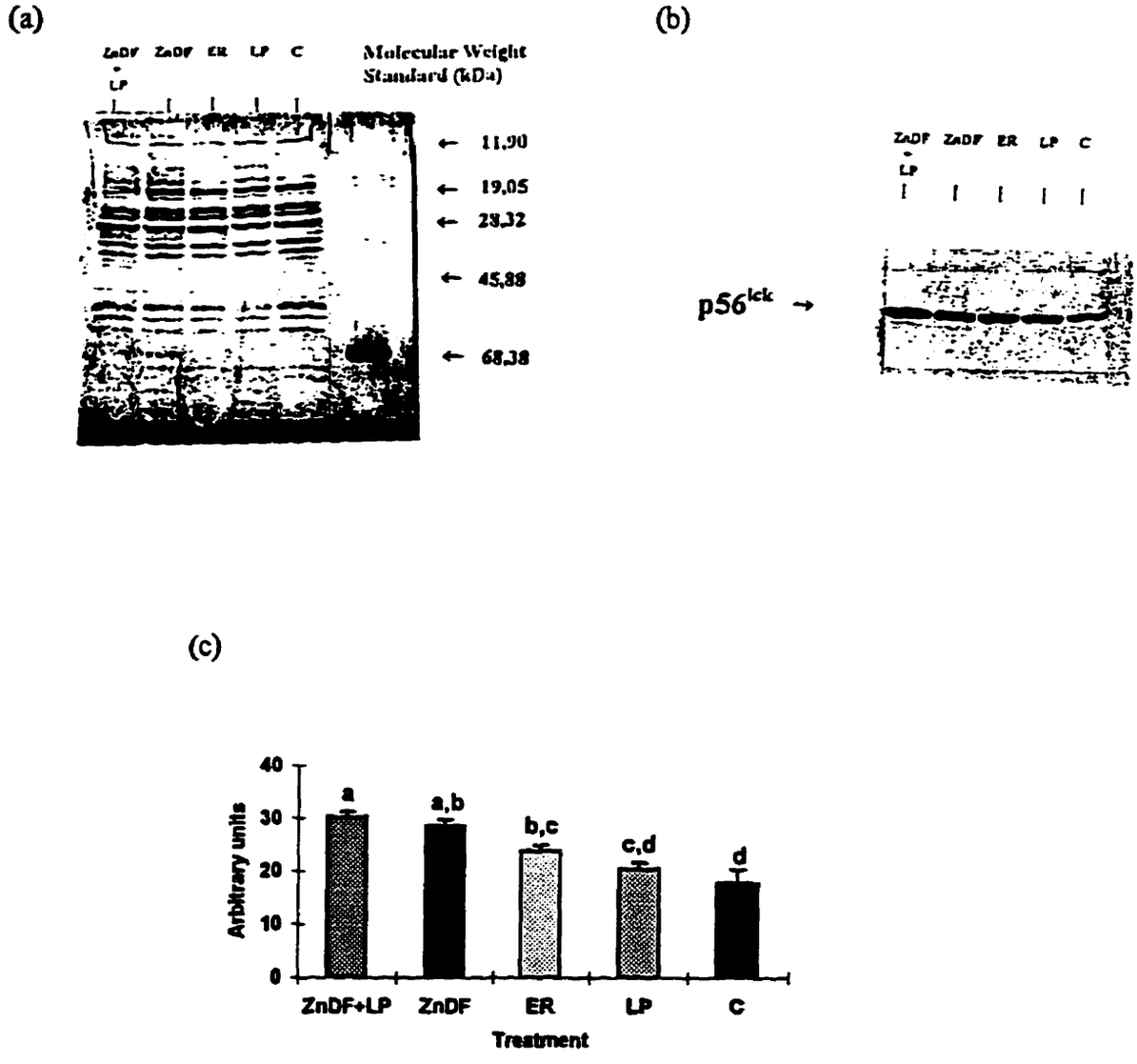

Figure 6. Effect of diet on expression of $p56^{lck}$. Western Immunoblotting for $p56^{lck}$ was performed on T lymphocyte cell lysates as described in Materials and Methods. An India ink stain of the nitrocellulose membrane (a) displays the elution of $p56^{lck}$ in comparison to the molecular weight standard and other proteins in the lysate. The expression of $p56^{lck}$ was detected by chemiluminescence (b). Arbitrary units for $p56^{lck}$ expression (c) were determined by image analysis scanning. The arbitrary units represent band area combined with grey intensity, and data was normalized by log transformation. Columns represent mean ± SEM, n=5. Lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

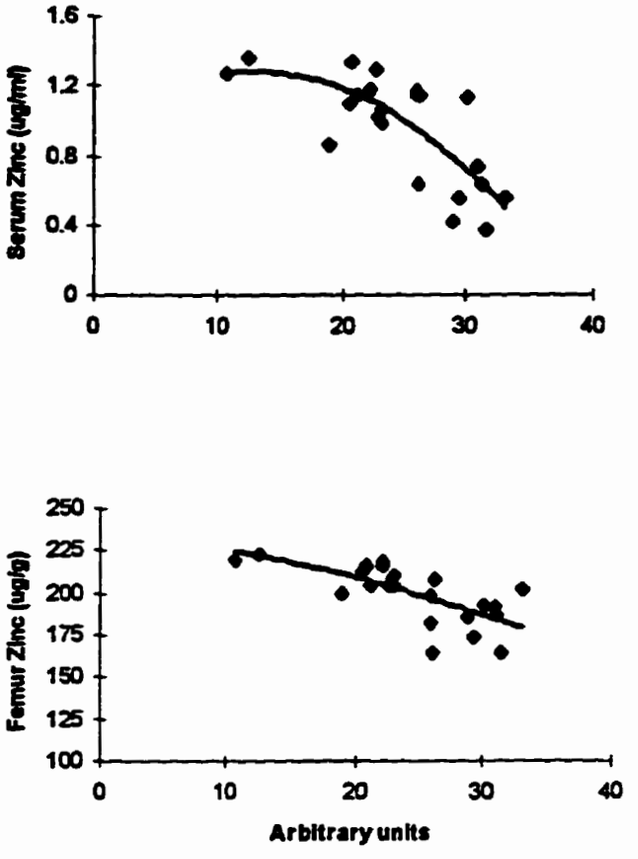

Figure 7. Scatter plot of zinc status vs the expression of $p56^{lck}$ in murine T-lymphocytes. Data points are values obtained from individual mice, n=25. Analysis by Pearson's correlation coefficient (r) revealed significant negative correlations between $p56^{lck}$ expression and (a) serum zinc concentration ($r = -0.71$, $p = 0.0002$) and femur (b) zinc concentration ($r = -0.70$, $p = 0.0002$).

V. Discussion

The models used in the current study were successful in inducing dietary zinc deficiency and malnutrition syndromes. This provided the means to investigate and compare the effects of these nutrient deficiencies on parameters reflective of zinc status and immunocompetence in an adult murine model. In retrospect, inclusion of an energy restricted group rather than a pair-fed group was beneficial since this provided a model for energy malnutrition. As a result of the success of the models, we were able to gain a better understanding of the relationship between zinc deficiency and two common types of malnutrition, and their effects on various parameters of zinc and immune status at the organ, cellular and molecular levels.

An important observation of the present study was that zinc deficiency can be associated with both protein and energy types of malnutrition. A diet deficient in zinc resulted in energy-type malnutrition, whereas dietary deficiencies of both zinc and protein resulted in protein-type malnutrition. Also interesting was the finding that splenocyte counts were equally depressed in the ER and ZnDF groups. In addition, protein deficiency appears to exert a protective effect against the adverse effects of zinc deficiency with regards to long term zinc stores and splenocyte counts. However, the most salient finding was at the molecular level, where dietary zinc deficiency appears to up-regulate the expression of $p56^{lck}$ in T lymphocytes. In fact, zinc status was negatively correlated with the expression of $p56^{lck}$. Each of these conclusions will be discussed in the following subsections.

This made possible the characterization of biochemical trends of the various forms of zinc deficiency and malnutrition in the adult murine model. In retrospect, inclusion of an energy restricted group rather than a pair-fed group was beneficial since this provided a model for energy malnutrition. As a result of the success of the models, we were able to gain a better understanding of the relationship between zinc deficiency and two common types of malnutrition, and their effects on various parameters of zinc and immune status at the organ, cellular and molecular levels.

Effects of zinc deficiency and malnutrition models on body weight

Groups receiving diets deficient in zinc, protein or energy did not experience any weight loss or gain although they weighed significantly less than the C group (Table 3). Since the mice were young adults, a small increase in weight (1.8g) was observed in the C group during the 4 week feeding trial. Also, likely due to their age, mice receiving dietary deficiencies did not experience weight loss. This is in contrast to weanling animals, where significant weight loss is commonly observed in nutritional deprivation.

Many investigations of the effects of dietary zinc or protein deficiency on immunocompetence have used weanling animals, and thus growth was a parameter of interest. Fraker et al (1991) reported an 18% decrease in body weight in 6 week old A/J mice receiving a zinc deficient diet (0.9 ppm) for a period of 4 weeks. Similarly, Petro (1985) observed a 29% decrease in body weight when 5 week old BALB/c mice were fed a diet containing 2% protein over a period of 3 weeks. These observations demonstrate that growth is an important factor which has an influence on outcome measurements in studies of nutritional deficiencies. Therefore, an adult animal model was chosen in order

to minimize some of the complications due to weight changes during periods of rapid growth.

It was surprising that the combined ZnDF + LP group had a similar final body weight compared to the ZnDF or LP groups, since a combined deficiency was anticipated to be a more severe dietary treatment than a single nutrient deficiency. A deficiency of both zinc and protein had the same effect on weight as did a single nutrient deficiency of zinc or protein. It appears that the effect of these nutrient deficiencies on body weight is not additive.

The trend of lower body weight seen in all groups of dietary deficiency in comparison to the C group is reflective of malnutrition and zinc deficiency syndromes, although further evidence regarding the type of malnutrition and presence of zinc deficiency is provided by biochemical assessment.

Evaluation of zinc status

This study revealed that dietary deficiencies of zinc, protein or energy can result in a decline in zinc status in young adult mice (4 months of age). Not surprisingly, groups fed zinc deficient diets (ZnDF and ZnDF + LP) displayed the largest decrease in zinc status, as indicated by significant depressions in serum zinc (48-50%↓) and femur zinc (15-21%↓) concentrations compared to the C group (Table 3). Previously, similar depressions in serum zinc concentration (51%↓) were observed in young (5-6 week old) C57BL/KsJ mice fed a zinc deficient diet (2 ppm zinc) compared to a control group fed a nutritionally adequate diet containing (30 ppm zinc) for a 4 week period (Donaldson et al, 1986). However, the reported decrease in femur zinc concentration in the dietary zinc

deficient group compared to the control group (44%↓) was greater than that observed in the current study (21.1%↓). This comparison demonstrates that young mice are more susceptible to a greater depletion of long term zinc stores than adult mice. This is not surprising since it is known that growth is a factor which increases the susceptibility to developing zinc deficiency (Aggett and Comerford, 1995).

Also, these observations illustrate that serum zinc levels do not necessarily correlate with femur zinc stores, since similar depressions of serum zinc seen in the young and adult mice do not result in the same depression in femur zinc stores. In agreement with this observation, Cook-Mills and Fraker (1992) have suggested that serum zinc is an unreliable indicator of the extent of zinc deficiency, since serum zinc levels were equally depressed in a study with both moderately and severely zinc deficient mice (Cook-Mills and Fraker, 1992).

It is noteworthy that the ZnDF group had a greater reduction in femur zinc concentration compared to the ZnDF+LP group. It appears that the single nutrient deficiency of zinc depletes long term zinc stores more quickly than a combined deficiency of zinc and protein, suggesting that protein deficiency may have a protective mechanism on zinc stores when zinc deficiency is present.

Although the low protein and energy restricted diets contained adequate zinc, mice receiving these dietary treatments demonstrated a marginal decline in zinc status as indicated by serum and femur zinc data (Table 3). This finding was expected since many reports have shown that malnutrition syndromes are associated with a decline in zinc status. Previous clinical trials of malnutrition have shown that both protein and energy malnutrition can adversely affect zinc status (Atalay et al, 1989). It has been suggested

that physiological changes in the intestinal mucosa brought about by malnutrition may adversely affect zinc absorption (Atalay et al, 1989). Others have suggested that low serum albumin concentrations may be associated with decreased serum zinc since albumin is the common carrier for zinc in the serum (Golden and Golden, 1979). Yet, this seems unlikely since serum zinc levels were similar in the ZnDF and ZnDF + LP groups, despite the greater depression in serum albumin concentration in the ZnDF + LP group.

The results of the current study demonstrate that a wide spectrum of zinc deficiency states were induced in all the treatment groups of dietary deficiencies. A more severe form of zinc deficiency was induced in animals receiving zinc deficient diets, whereas energy restriction and diets deficient in protein resulted in a mild form of zinc deficiency. Presently in the clinical setting, there are no precise biochemical markers reflective of the severity of zinc deficiency, although experimentally, differences in severity of zinc deficiency can be identified by comparative analysis of femur zinc concentration. Due to a lack of understanding of zinc homeostasis and inability to assign biological values to various levels of zinc deficiency, terms such as severe zinc deficiency and moderate zinc deficiency are purely qualitative at this time.

Micronutrient malnutrition is being increasingly recognized as a global health problem. Micronutrient enrichment of food staples through plant breeding is currently underway (Bouis, 1996). The concept of lowering the incidence of micronutrient malnutrition through plant breeding is appealing since plants and cereals are considered to be staple foods worldwide, and they tend to be of poor nutrient bioavailability due to the presence of inhibitory compounds such as phytate. Although it is not recommended to produce plants of lower phytate content, due to the importance for phytate in plant

structure, the ideal breeding strategy would be to increase the concentration of amino acids (cysteine, lysine and methionine) which increase mineral bioavailability (Bouis, 1996). Presently, zinc enriched wheat varieties have been developed and are now grown commercially in Australia (Bouis, 1996).

Identification of the different forms of malnutrition:

The results of the current study demonstrate that different forms of malnutrition were induced as evidenced by serum albumin and liver lipid data (Table 3). The distinguishing features of protein malnutrition are depressed serum albumin and increased liver lipid concentration (Edozien, 1968). In this study, animals receiving low protein diets, namely the LP and ZnDF + LP groups, exhibited similar decreases in serum albumin and increases in liver lipid concentrations compared to the C group. Not only do these results demonstrate that protein malnutrition was induced in these groups, but also that the extent of malnutrition was similar. It appears that a diet deficient in both zinc and protein, does not result in a more severe form of protein malnutrition than dietary protein deficiency alone.

The decrease in weight in the ZnDF and ER groups in the absence of biochemical features of protein deficiency suggest that energy-type malnutrition (marasmus) was induced. In experimental animals, energy malnutrition can be characterized by weight loss and normal or slightly decreased serum protein and liver lipid concentrations (Heard et al, 1977). An interesting finding in this experiment was that the ER group experienced a significant increase in serum albumin concentration compared to the C group. A reason for this may be that the ER group may have been dehydrated, due to daily fasting,

including a 24 hour fast prior to termination. Dehydration has the effect of "concentrating" serological parameters, therefore distorting the actual values. It has been previously shown that rats receiving a nutritionally adequate diet, but fasted for 48 hours, results in elevated serological parameters, suggesting that these animals experienced some degree of dehydration (Heard et al, 1977).

These results also demonstrate that zinc deficiency can be associated with both protein and energy malnutrition. The induction of energy malnutrition in animals receiving a diet deficient in zinc alone may be caused by a concomitant decrease in energy intake resulting from zinc deficiency related anorexia. Although feed intake was not recorded in the present study, zinc deficiency related anorexia is a phenomenon that has been demonstrated in numerous clinical trials and experiments. Cook-Mills and Fraker (1993) have shown that feed intake in mice receiving a zinc deficient diet was significantly reduced (21%↓) compared to mice receiving a zinc adequate diet *ad_libitum*. Also in this study protein malnutrition was induced when mice were fed a diet deficient in both zinc and protein. Although zinc deficiency can be associated with both protein or energy malnutrition, dietary deficiencies in protein or energy seem to be the determining factors in the type of malnutrition produced. It appears that the type of malnutrition is more closely related to the macronutrient deficiency rather than micronutrient deficiency.

Assessment of immunological parameters:

The data from the current study demonstrate that diets deficient in zinc, protein or energy can induce depressions in spleen weight and splenocyte counts. Atrophy of the

spleen and depressed lymphocyte counts are demonstrated features of experimental zinc deficiency and malnutrition syndromes (Hansen et al, 1982). However, until now, the effects of these various nutritional deficiencies on immunological parameters have not been investigated simultaneously.

All groups receiving nutritionally inadequate diets in zinc, protein or energy experienced a decrease in spleen weight compared to the C group. The ER and LP groups experienced a greater depression in spleen weight than the ZnDF and ZnDF+LP groups (Table 4). Two observations can be made based on this data. Firstly, deficiencies in protein and energy induce a greater extent of splenic atrophy than do dietary deficiencies of zinc. Secondly, since the LP group experienced a greater decrease in spleen weight than the combined ZnDF+LP group, this suggests that a combined deficiency of both macronutrient and micronutrient may be less detrimental than a single (unbalanced) macronutrient deficiency on spleen weight.

Splenocyte counts (total count/spleen, Table 4) were reduced in all groups of dietary deficiency, with the ER and LP groups displaying the greatest decrease. This decrease appears to be reflective of the significant splenic atrophy observed in these two groups. Although total splenocyte counts were significantly reduced in the ZnDF+LP and LP groups compared to the C group, when expressed as splenocytes/mg spleen, splenocyte counts are not significantly different from the C group. It has been suggested that damage to the supporting cell system (Hassall's corpusules) in lymphoid organs may be partially responsible for the atrophy observed in protein-energy malnutrition (Woodward, 1992). It may be possible that the splenic atrophy observed in nutritional deficiencies of zinc, protein or energy may be due to different mechanisms.

The observation of equally depressed splenocyte counts in the ZnDF and ER groups is contrary to previous studies, where the dietary zinc deficient group had a greater reduction in spleen cell counts and body weights were 19% less than their pair-fed counterparts (Cook-Mills and Fraker, 1992). In the present experiment, the energy restricted group was used in order to achieve the same body weight observed in the zinc deficient group whereas others most often pair-fed in order to account for differences due to the zinc deficiency related anorexia. These results indicate that energy restriction has a significant effect on splenocyte counts.

Expression of $p56^{lck}$ in T lymphocytes

The most interesting finding in this study was that dietary zinc deficiency appears to play a role in up-regulating the expression of $p56^{lck}$ in murine T lymphocytes. The observation of the high expression in $p56^{lck}$ in dietary zinc deficiency, particularly in the conbined zinc and protein deficient diet, was unexpected (Figure 6). In fact, mice from the ER and LP groups, which displayed a decrease in zinc status, also demonstrated elevated expression of $p56^{lck}$ compared to the C group. Correlation analysis revealed that the expression of $p56^{lck}$ with serum and femur zinc concentrations were negatively correlated (Figure 7). It appears that as zinc status declines, the expression of $p56^{lck}$ is heightened.

The expression of T lymphocyte $p56^{lck}$ may potentially be a serological parameter for clinical assessment of immune function and zinc status, although its assessment in the laboratory would be technically involved. However, before this parameter can be used in a clinical setting, several issues need to be resolved. It remains to be determined whether

the expression of $p56^{lck}$ in the spleen is reflective of that in the serum in order to examine the expression of $p56^{lck}$ using blood samples (circulating T-lymphocytes). Also, it is unknown whether the expression of $p56^{lck}$ in human T-lymphocytes is reflective of deficits in nutritional status (zinc, protein or energy) as was observed in the murine splenic T lymphocytes. The use of a particular intracellular index may lead to the development of a sensitive index of zinc and immune status. Presently, there is a lack of a sensitive index for zinc, since serum zinc levels are known to be affected by stressful conditions, including infection (Prasad, 1995).

The significance of increased expression of $p56^{lck}$ remains to be determined. Does an increase in expression of $p56^{lck}$ correlate with an increase in function, and if so, is an increase in function beneficial or detrimental? It would be of interest to determine the activity of $p56^{lck}$ directly by assessment of the active form of $p56^{lck}$ (phosphorylation of Tyrosine residue 394) following T cell receptor and CD4 stimulation. Alternatively, the activity of protein tyrosine kinases following $TCR/CD3^{+}$ and $CD4^{+}$ co-stimulation could be detected by a radioactive assay of ^{32}P incorporation. $p56^{lck}$ function may be evaluated indirectly by assessment of downstream biochemical events following T lymphocyte activation. Such indices include calcium mobilization, phosphorylation of other signaling proteins such as PKC, and cell proliferation.

A possible explanation for this heightened expression may be that $p56^{lck}$ is conserved since it plays a pivotal role in the T lymphocyte signal transduction pathway. A state of zinc deficiency may induce the increase in expression of $p56^{lck}$ by a negative feedback mechanism at the transcriptional level. A relationship between dietary zinc deficiency and gene expression has been proposed, although zinc deficiency was thought

to restrict gene expression, since zinc is known to be an integral component of zinc finger domains of transcription factors. Needless to say, our current understanding of zinc and gene expression is limited, although the findings of the current study shed new light on this interaction. Another possible mechanism leading to increased expression of $p56^{lck}$ may be at the post-translational level, where changes in $p56^{lck}$ may occur which affect the rate of protein turnover, leading to an accumulation of $p56^{lck}$.

Increases in the expression and function of $p56^{lck}$ may be detrimental. Cell lines transfected with an active form of $p56^{lck}$ induced apoptosis, whereas cell lines defective for $p56^{lck}$ were resistant to apoptosis (Maddalena et al, 1995). The hypothesis that overexpression of $p56^{lck}$ would promote apoptosis may explain the depression of lymphocytes seen in zinc deficiency. The theory of increased apoptosis of lymphocytes in zinc deficiency has been proposed by Fraker (1996).

Other deleterious effects of increased expression of $p56^{lck}$ have been reported. Perlmutter (1993) demonstrated that a 4-5 fold increase in expression of $p56^{lck}$, in transgenic mice compared to control mice, provokes the development of thymic tumors, which were found to consist of immature T lymphocytes.

The evidence of up-regulated expression of a signal transduction protein in various zinc and malnutrition syndromes in the current experiment substantiates prior speculations of the paradoxical phenomena of immune suppression occurring concomitantly with hyperimmune responses. Taylor et al (1997) demonstrated that splenocytes from mice receiving protein deficient diets exhibited an overshoot in proliferative response when placed in a complete thiol-supplemented media, compared to control mice. In humans, a similar phenomena is observed in patients with HIV who

display immunodeficiency, while exhibiting hyper-immune activation, indicated by increased expression of lymphocyte surface markers and spontaneous cytokine secretion (Oyaizu and Pahwa, 1995). Because increases in expression of $p56^{lck}$ were observed in various nutritional deficiencies (zinc, protein and energy), it remains to be determined whether this index could distinguish if the immunodeficiency was due to zinc deficiency or malnutrition.

In conclusion, the present study suggests that dietary zinc deficiency, varying in severity can increase the expression of $p56^{lck}$ in murine T lymphocytes, though the effects of this increase and the underlying mechanisms responsible for this increase remain to be elucidated.

VI. Summary & Conclusion

The objective of the current experiment was to investigate the effects of dietary zinc deficiency and malnutrition syndromes (protein deficiency, combined zinc and protein deficiency, and energy restriction) on the expression of $p56^{lck}$, an early T lymphocyte signal transduction zinc finger protein. This was investigated in order to elucidate molecular mechanisms involved in the development of zinc deficiency pathology and to distinguish them from molecular events resulting from various forms of malnutrition.

In order to understand these syndromes in their entirety, the effects of the various dietary treatments on indices of zinc and immune status were identified and related to events occurring at the molecular level. Immunologically mature adult murine models of zinc deficiency and malnutrition syndromes were developed in order to study these parameters.

In the current experiment, zinc deficiency and malnutrition syndromes were sucessfully induced. Mice receiving diets low in protein developed protein malnutrition, whereas mice consuming a diet deficient in energy, either by energy restriction or zinc deficiency related anorexia, experienced energy type malnutrition. Dietary zinc deficiency was shown to result in a significant decline in zinc status, although slight depressions in long term zinc stores were observed in mice receiving zinc adequate low protein or energy restricted diets. These observations demonstrate that a deficiency in protein or energy can also resulted in some degree of zinc deficiency in this murine model of protein or energy malnutrition. In addition, although zinc deficiency can be associated with both

protein or energy malnutrition, dietary deficiencies in protein or energy seem to be the determining factors in the type of malnutrition produced.

Decreases in spleen weight and total splenocyte counts were observed in all deficient groups, although the largest decreases were observed in mice fed the energy restricted or low protein diet. The observation of the greater depression in these parameters in the ER group compared to the ZnDF group was contrary to previous studies of zinc deficiency (Cook-Mills and Fraker, 1993). This is likely attributable to the fact that the energy restricted group was pair-weighed in order to control for weight loss rather than pair-fed to control for reduced feed intake. Consequently, the ER group experienced a greater degree of weight loss than mice in previous studies which were pair fed to their zinc deficient counterparts (Cook-Mills and Fraker, 1993; Donaldson et al, 1986).

It appears that combined protein and zinc deficiency provides a protective effect against the adverse effects of zinc deficiency on long term zinc stores and splenocyte counts. In addition, the combined zinc and protein deficiencies were not shown to result in a greater decline in weight than single nutrient deficiencies of zinc or protein. Perhaps a combined zinc and protein deficient diet represents a "balance" of both macronutrient and micronutrient deficiencies which may be better tolerated by homeostatic mechanisms compared to a single, unbalanced nutrient deficiency. Although single dietary micro/macronutrient deficiencies are uncommon, it is interesting to observe their effects on various indices of nutritional and immunological status.

The most significant finding of this study was that zinc deficiency appears to up-regulate the expression of $p56^{lck}$, although it is not known whether this is a protective

mechanism or a molecular insult which may contribute to immune dysfunction. It is possible that nutrient-gene interactions exist which promote the over-expression of proteins which are at pivotal sites in metabolic pathways, such as $p56^{lck}$. Alternatively, heightened expression of $p56^{lck}$ may be detrimental as it may induce apoptosis of lymphocytes and the development of thymic tumors resulting in immature T lymphocytes (Perlmutter, 1993). Further research is needed in order to provide information of the roles of $p56^{lck}$ in transducing signals that lead to cell proliferation as well as apoptosis. An understanding of these roles will help in determining the consequences of increased expression of $p56^{lck}$ in T lymphocytes.

Presently, cellular function is assessed indirectly by tests which evaluate cellular response. With advances in technology, the focus of research can now be the investigation of particular cellular and molecular events, such as signal transduction and zinc finger proteins, and how they may be affected by nutrient deficiency. Understanding these issues will require the direct investigation of the interaction of zinc and amino acid residues in zinc finger domains during nutritional deprivation. Also, it would be of interest to elucidate the mechanisms by which a nutrient deficiency of zinc affects gene expression of $p56^{lck}$ and other proteins found within signal transduction pathways.

A better understanding of the molecular events which are adversely affected by nutrient deficiencies may lead to the development of not only tests of immunocompetence, but also indices of nutritional status. Molecular insults in immune cells may be sensitive indicators of an early decline in nutritional status since cells of rapid turnover are quickly affected by nutritional deficiencies.

Investigations of zinc repletion and restoration of nutritional status following a period of zinc deficiency and malnutrition may provide information on whether improved zinc status leads to reversal of the heightened expression of $p56^{lck}$ and improvements in immunological status. Furthermore, such studies would provide the framework for developing protocols for zinc supplementation which might be beneficial for the immuno-compromised.

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Appendix A

Method Development

A method for murine lymphocyte isolation suitable for subsequent analysis of the signal transduction protein $p56^{lck}$ by Western Immunoblotting

Many experiments investigating the in vitro functioning of T lymphocyte signal transduction proteins via Western Immunoblotting have been conducted using T cell lines (Eljaatari et al, 1995 and Kanner et al 1992). In contrast, the effects of dietary manipulations on the in vivo expression of T lymphocyte signal transduction proteins have not been elucidated using an animal model. Therefore, a method for the isolation and preparation of lymphocytes from a suitable organ for Western Immunoblotting analysis was needed.

The spleen, along with other lymphoid organs, harbors B and T lymphocytes as well as accessory cells which together cooperate in the generation of the immune response. The spleen has been extensively used to study immune cell populations and their function since it is a principal site of immunologic response to blood borne antigens (Abbas et al, 1991). Furthermore, the large size of the spleen and subsequently large immune cell populations in comparison with smaller lymphoid organs, such as lymph nodes, make the spleen an organ of choice for cell isolation and analysis.

The murine immune system has served many researchers as a model of mammalian immune function. Consequently, many antibodies used for investigating immune function

have been developed in the mouse. It is for these reasons that the murine spleen was chosen for analysis. The following discussion is a summary of the events leading to the optimized protocol for lymphocyte preparation and Western Immunoblotting. Therefore, the specifics of reagents, buffers and equipment that were used in the optimized protocol will be detailed in Methods and Materials.

Since the protein of interest, $p56^{lck}$, is principally found in T lymphocytes, and antibody targeted specifically to $p56^{lck}$ was to be used for Western Immunoblotting, the presence of other mononuclear cells such as B lymphocytes and macrophages was thought to not interfere with $p56^{lck}$ analysis. Therefore, it was anticipated that a splenocyte culture comprising mononuclear cells would be sufficient and that T lymphocyte purification would be unnecessary.

C57BL/6 mice used in the optimization of the lymphocyte preparation were purchased from the University of Manitoba animal care facility. Mice were weighed, then terminated by $C0_2$ asphyxiation and cervical dislocation according to the Canadian Council on Animal Care Guidelines. The spleens were removed aseptically, weighed then frozen at -80^0C. A spleen cell suspension was prepared under aseptic conditions. Sterile bent needles were used to scrape spleen cells into RPMI 1640 media (GIBCO, Grand Island, NY), creating a total spleen suspension containing mononuclear and polyphomornuclear cells, erythrocytes and dead cells.

Splenocytes were isolated from the total spleen cell suspension by gradient centrifugation using Lympholyte M (Cedarlane Laboratories, Hornby, ON). After centrifugation at 400 x G for 20 minutes, the splenocyte layer was washed in RPMI 1640 and cells were counted in a hemacytometer. Unfortunately, not a single cell was present.

Subsequent trials with fresh, unfrozen spleen proved to be more fruitful in isolating a viable mononuclear suspension, as an abundance of cells was visualized under the microscope.

After counting the mononuclear fraction from fresh, unfrozen spleen, cells were pelleted and resuspended in 3 mls of RPMI 1640 media. The protein concentration of the suspension was determined with the BCA Protein Assay Reagent. Cell suspensions were brought to a concentration of 1 ug/ul with RPMI 1640 media. Five ug (5 ul) of protein was mixed with an equal volume of 2 x sodium dodecyl sulphate (SDS) sample buffer then boiled for 3 minutes in order to partially denature and solubilize proteins.

Difficulties in loading the samples onto the gel for subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were encountered. After heating, the samples had become extremely viscous and accurate pipetting was impossible. Nonetheless, various amounts of sample were loaded and proteins were separated according to molecular weight by SDS-PAGE using a 10% separating gel and 5% stacking gel. A 10% gel has been used previously to analyze $p56^{lck}$ (molecular weight = 56 kDa) since its porosity is best suited for separation of proteins of medium molecular weight (40-80 kDa).

Separated proteins were then transferred onto a nitrocellulose membrane, blocked in 0.5% block solution and probed with the primary antibody, polyclonal anti-lck kinase (1: 1000 dilution). Peroxidase-labeled anti-mouse/rabbit IgG (1:1000 dilution) was used as the secondary antibody. This primary-secondary immune complex was detected on Kodak film by chemiluminescence, but unfortunately, no bands were detected. An India ink stain of the membrane indicated that transfer of proteins from the gel to the membrane

did occur, however, bands were diffuse rather than the crisp bands seen in the positive control (Figure 4a, p.86). Two problems, that of sample viscosity and protein separation by SDS-PAGE needed to be resolved.

Firstly, trials examining the effect of heat on the viscosity of the sample were conducted in the laboratory. Splenocyte suspensions were prepared as described above in 2 x SDS sample buffer, however some samples were boiled for 3 minutes whereas others were not. The viscosity of the heated samples greatly exceeded that of the non heat treated samples. This could be explained by considering the structure of a lymphocyte, where the nucleus is surrounded by a thin layer of cytoplasm. The DNA found within lymphocytes may not be solubilized as easily as cytoplasmic proteins, since heat treatment will denature the long strands of DNA causing clumping and creating increased viscosity. Further processing was indicated due to the problems with viscosity and hopefully, further modifications would improve protein separation by SDS-PAGE.

Elimination of DNA clumping in the sample could be achieved by removal of the nuclear fraction of the cell. Cells can be lysed chemically with a lysis buffer (Franco et al, 1995; Park et al, 1991) or mechanically by homogenization (Pernelle et al, 1991), both in the presence of protease inhibitors to prevent proteolysis. The nuclear fraction is then pelleted after centrifugation, leaving cytosolic proteins in the supernatant. Others have incorporated a DNA shearing step by passing the sample through a 27 gauge, narrow bore needle after cellular lysis with RIPA buffer (Grossman et al, 1995). The shearing of DNA would result in a more complete separation of cytosolic and nuclear fractions after centrifugation.

Once again, Western blotting for $p56^{lck}$ was attempted by isolating splenocytes using Lympholyte M as described above. Cellular lysis was achieved by incubating cells for 30 min in ice cold RIPA buffer containing protease inhibitors (Kanner et al, 1990). Cell lysates were then passed through a 27 G needle, then microcentrifuged at 10 000 rpm for 10 minutes. Determination of protein concentration, heat treatment, SDS-PAGE and Western Immunoblotting were as described above, and yet no bands could be detected for $p56^{lck}$. However, on a positive note, sample viscosity following heating had been eliminated by the removal of the nuclear fraction. In addition, proper protein separation had occurred, as indicated by the sharp crisp bands of the India ink stained membrane (Figure 4b, p.86).

Attempts at optimizing the detection of $p56^{lck}$ were now focused on Western Immunoblotting conditions, rather than cell preparation. Varying dilutions of primary and secondary antibodies were tried. Likewise, alterations in duration of antibody washing steps and membrane blocking were made, yet $p56^{lck}$ remained undetected.

A potential problem causing the absence of detection of $p56^{lck}$ could stem from a lack of adequate amounts of the protein required for its detection. Since $p56^{lck}$ is mainly found in T lymphocytes, contributions of macrophages and B lymphocytes to the protein concentration of the cell lysate could in fact "dilute" $p56^{lck}$. Therefore, isolation of T lymphocytes would increase the amount of $p56^{lck}$ in the lysate. This would also eliminate the B lymphocyte contributions of $p56^{lck}$ since these cells have been found to contain low amounts of the protein (Gold and Defranco, 1994), and thus this approach would provide an analysis of $p56^{lck}$ exclusively in the T lymphocyte.

T lymphocytes were isolated using a mouse T cell enrichment immunocolumn. A splenocyte suspension was prepared using Lympholyte M described as above and 1 x 10^8 cells adjusted to 5 x 10^7 cells/ml were then run through the column at a flow rate of 6-8 drops/minute. The eluate from a mouse T cell immunocolumn contains an enriched T cell population, while unwanted cells such as B cells, monocytes and macrophages remain bound in the column matrix. The eluant was centrifuged to pellet T lymphocytes, then cells were counted using a hemacytometer. Proper elution of T lymphocytes did not occur as only 1% (expected 15-25%) of cells were eluted.

Suggestions as outlined by the manufacturer (Biotex Laboratories, Edmonton, AB) such as adding 2% fetal calf serum to the wash buffer, and increasing flow rate were tried in attempt to maximize T cell elution. The percent of cells eluted increased to 7% , however B lymphocytes may also have been eluted due to an increased flow rate. Although viable splenocyte suspension was being prepared, these cells were not being properly eluted from the column. Modifications to the splenocyte preparation was warranted.

A method used to isolate spleen cells was provided by Biotex Laboratories as they suspected that cells isolated by Lympholyte M may adhere to the column. The method involved the lysis of erythrocytes with 0.19 mol/L Tris-Ammonium Chloride, whereas with Lympholyte M erythrocytes were pelleted by centrifugation. The splenocyte suspension prepared by Tris Ammonium Chloride treatment was washed, counted and applied to the mouse T column as described above. Recovery of sufficient amounts of viable T lymphocytes, as determined by Trypan blue exclusion, was achieved.

Characterization of the T lymphocyte sub-populations $CD4^+$ (T helper) and $CD8^+$ (T cytotoxic), as well as T cell receptor $CD3^+$ distribution were determined on stock C56BL/6 mice by flow cytometry in order to verify the purity of the preparation obtained from the Mouse T cell immunocolumn according to the protocol provided by the manufacturer. Fluorescent antibodies (Cedarlane Laboratories, Hornby, ON) were used for labeling cell markers (ie. CD4, CD8 and CD3). Briefly, cells were incubated with either anti-CD4 (Tri-color anti-mouse CD4 monoclonal antibody, CL 013TC) or anti-CD8 (PE anti-mouse Ly 2 CD8a monoclonal antibody CL 169PE), along with anti-CD3 (FITC anti-mouse T3 complex CD3ε monoclonal antibody CL 7202F). Fluorescently labeled cells were then detected by their characteristic fluorescence emission. Greater than 95% of the cells were $CD3^+$. Of these 55% and 37% of the cells were $CD3^+/CD4^+$ and $CD3^+/CD8^+$, respectively, which is representative of normal T lymphocyte sub-populations. Approximately 65% of T lymphocytes express CD4 and 35% express CD8 (Abbas et al, 1991).

Following T lymphocyte isolation by the immunocolumn, cells were subsequently lysed and the cytosolic fraction isolated and analyzed for $p56^{lck}$ by SDS-PAGE followed by Western Immunoblotting as described earlier. Non-specific binding of antibody to most of the protein bands was detected on the film (Figure 4c, p.86). Efforts to rectify this problem such as increasing primary and secondary antibody dilutions, as well as antibody washings were unsuccessful.

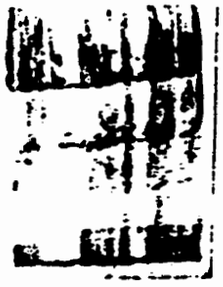
Immunoprecipitation, a means of purification of a specific antigen was tried in order to identify $p56^{lck}$ by Western Immunoblotting. This would potentially reduce all non-specific binding. Other researchers have successfully immunoprecipitated $p56^{lck}$ in

cell lines (Pernelle et al, 1991). The protocol involves the binding of anti-$p56^{lck}$ antibody to the specified antigen, $p56^{lck}$ (N terminal domain) in the cell lysate. The immune complex is then captured by protein A sepharose beads and precipitated by centrifugation. Unbound antigens are removed by washing with RIPA buffer and the protein of interest is released from the protein A sepharose beads by antibody denaturation in boiling SDS sample buffer. After SDS-PAGE and Western Immunoblotting, bands appeared at the molecular weight between 44-87 kDa, as indicated by the molecular weight standard.

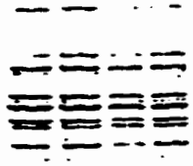
The positive control (a cell lysate prepared from human Jurkat cells containing $p56^{lck}$) sent along with the antibody (Upstate Biotechnologies Incorporated, Lake Placid, NY) also appeared to be successfully immunoprecipitated, as detected on the film. A sample blank, solely containing RIPA buffer was immunoprecipitated in order to verify these results. Surprisingly, the sample blank produced a band at the same molecular weight as the sample and positive control (Figure 4d, p.86), indicating that the antibody used in immunoprecipitation, particularly the heavy chain (molecular weight = 55 kDa, confirmed by the manufacturer) was being resolved by electrophoresis and detected by Western Immunoblotting. It appears that the heavy chain of the antibody had become dissociated from the whole Immunoglobulin when subjected to heat and SDS sample buffer.

Immunoprecipitating $p56^{lck}$ under denaturing conditions may produce confounding results since $p56^{lck}$ and the disassociated antibody heavy chains, of molecular weights 56 kDa and 55 kDa respectively, may be resolved together. This raises concerns for published studies of immunoprecipitation of $p56^{lck}$ under denaturing conditions.

(a)



(b)



(c)

(d)

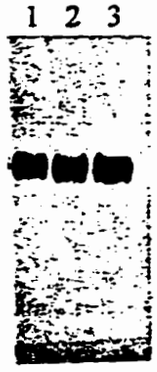


Figure 4. India ink stains and Western blots illustrating the development of the method used for Western Immunoblotting of $p56^{lck}$ in splenic T lymphocytes. India ink stain of the blot of the blot from unlysed (a) and lysed (b) splenic T lymphocytes. Difficulties in non-specific binding were encountered as indicated by the film (c). Immunoprecipitation was shown to be unsuccessful, since a sample blank [lane 1] produced a similar band on the film compared to the positive control [lane 2] and the sample [lane 3] (d).

These concerns were raised with the manufacturer, and they questioned the viability of the antibody. A new vial was sent, and probing of T lymphocyte lysates and the positive control for $p56^{lck}$, was accomplished without immunoprecipitation. It appeared that previous trials of Western Immunoblotting were unsuccessful due to a non-viable antibody.

In summary, the optimized procedure is as follows:

Isolation of spleen cells in PBS/2% FCS

↓

Lysis of red blood cells with Tris-Ammonium Chloride

↓

Total mononuclear cell count

↓

T lymphocyte isolation and count (T cell immunocolumn)

↓

T lymphocyte lysis

↓

Determination of T lysate protein concentration

↓

Western Immunoblotting and India Ink staining of membrane

Appendix B

Effects of dietary treatments on body and liver weights, and various biochemical parameters of zinc and protein status in mice[1]

	Treatment group[2]					
	B	ZnDF + LP	ZnDF	ER	LP	C
Body weight *(g)*	22.2^{B} ± 0.31	21.2^{B} ± 0.50	21.6^{B} ± 0.86	21.2^{B} ± 0.31	20.5^{B} ± 0.56	24.8^{A} ± 0.53
Serum Zn *(µg/ml)*	1.03^{B} ± 0.09	0.59^{C} ± 0.03	0.64^{C} ± 0.10	$1.12^{A,B}$ ± 0.04	1.01^{B} ± 0.03	1.23^{A} ± 0.03
Femur Zn *(µg/g dry wt)*	194^{C} ± 3.55	189^{C} ± 3.95	175^{D} ± 4.55	203^{B} ± 2.13	207^{B} ± 1.38	221^{A} ± 1.42
Lipid conc. *(mg/g* liver)	38.1^{C} ± 0.03	74.1^{A} ± 0.44	53.1^{B} ± 0.07	60^{B} ± 0.16	72.9^{A} ± 0.41	56.3^{B} ± 0.13
Liver weight *(g)*	1.28^{A} ± 0.02	0.90^{C} ± 0.04	1.10^{B} ± 0.06	$1.17^{A,B}$ ± 0.07	0.93^{C} ± 0.03	1.28^{A} ± 0.05
% Liver/Body weight	5.76^{A} ± 0.06	4.24^{C} ± 0.21	5.09^{B} ± 0.15	$5.51^{A,B}$ ± 0.28	4.52^{C} ± 0.11	5.16^{B} ± 0.16
Serum albumin *(µmol/l)*	568^{A} ± 15.24	430^{C} ± 15.44	501^{B} ± 9.31	576^{A} ± 18.37	438^{C} ± 14.30	450^{C} ± 11.39

[1]Values are means ± standard error of the mean, n = 9 except for B, ZnDF + LP and LP where n = 5, n = 7 and n = 8, respectively. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] B = Baseline, ZnDF+LP = Zn deficient + 2% Protein, ZnDF = Zn deficient, ER = Energy Restriction, LP = 2% Protein, C = Control.

Effect of dietary treatment on spleen weight and total splenocyte count[1]

	Treatment group[2]					
	B	ZnDF+ LP	ZnDF	ER	LP	C
Spleen *mg*	89.1^{A} ± 10.00	$72.9^{C,B}$ ± 3.20	75.8^{B} ± 6.80	58.5^{D} ± 3.10	$60.9^{C,D}$ ± 4.10	101.1^{A} ± 2.50
% Spleen/body weight	$0.40^{A,B}$ ± 0.011	0.35^{B} ± 0.019	0.35^{B} ± 0.024	0.28^{C} ± 0.012	0.29^{C} ± 0.017	0.41^{A} ± 0.015
Splenocytes per spleen, x 10^{8}	2.44^{A} ± 0.10	1.40^{C} ± 0.12	$1.12^{C,D}$ ± 0.12	0.84^{E} ± 0.04	$1.04^{D,E}$ ± 0.11	1.93^{B} ± 0.13
Splenocytes (x10^{6}) per mg spleen	2.74^{A} ± 0.10	1.91^{B} ± 0.14	1.56^{C} ±0.06	1.44^{C} ± 0.03	$1.68^{B,C}$ ± 0.11	1.91^{B} ± 0.13

[1]Values are means ± standard error of the mean, n=9, except for the B, ZnDF+LP and LP groups where n=5, n=7 and n=8 respectively. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] B=Baseline, ZnDF+LP = zinc deficient + 2% Protein, ZnDF = Zn deficient, ER = Energy Restriction, LP = 2% protein, C = Control.