The effect of zinc deficiency on the production of inflammatory cytokines from murine macrophages

by

Haa-Yung Lee

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Microbiology, Immunology and Preventive Medicine
Interdepartmental Major: Immunobiology

Approved:

Members of the Committee

Iowa State University
Ames, Iowa

1993

TABLE OF CONTENTS

GENERAL INTRODUCTION

Malnutrition is one of the world-wide problems. Underdeveloped countries have Protein-Calorie Malnutrition (PCM) endemics but developed countries have selective element malnutrition problems among their population. One of the elements of concern is zinc. Zinc has been known as an essential trace element in organisms. Its importance in normal biochemical and physiological processes has been studied by many researchers. Another interesting aspect is the similarity of symptoms between PCM and zinc deficiency.

Zinc also acts as a critical element in immunological processes in animals. A variety of immune dysfunction associated with zinc deficiency has been reported. Some of those are caused by a congenital zinc malabsorption syndrome, acrodermatitis enteropathica, and a portion of the patients who suffers inflammatory bowel disease has common symptoms. Generally, in zinc deficient state, animals show characteristic features such as growth retardation, hepatomegaly, skin lesions, diarrhea, anorexia, impaired wound healing, and recurrent infections. These symptoms are very similar to those of chronic inflammatory disease. Cytokines, especially tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6), are putative mediators of inflammatory disease. They are released from monocytes or macrophages as a primary source.

The overall objective of this work is to understand the relationship between zinc deficiency and cytokine production by murine macrophages.

Explanation of Thesis Format

This thesis includes one manuscript. The manuscript is written in the American Society for Microbiology style (Infection and Immunity). A literature review precedes the manuscript and a general summary follows the manuscript. The literature cited for the literature review is listed after the general summary.

LITERATURE REVIEW

General Background of Nutrition in Immunity

Malnutrition has been one of the most significant world-wide concerns. In Third World countries, tens of millions of people suffer from hunger due to food shortages and thousands die daily of starvation and related diseases (2, 18, 20, 90). However, malnutrition is not a problem restricted to poor countries. Even in wealthy countries, it is encountered among infants, children, and the elderly of poor families (11, 18, 22, 64). It is also a very common problem among alcoholics, drug addicts, and those who suffer from anorexia nervosa (54, 96). In cases of long-term hospitalization, patients are vulnerable to malnutrition, especially when receiving parenteral feeding (56, 106). Malnutrition may also arise in accordance with physiological conditions such as impaired intestinal absorption, utilization, and excessive loss of selective nutrients (110). In tropical regions, a malabsorption syndrome, like tropical sprue, can induce temporary or chronic malnutrition by damaging the digestive tract (87).

Among the many nutritional deficiencies, Protein-Calorie Malnutrition (PCM), or Protein-Energy Malnutrition (PEM) is the most devastating problem. In Biafra, Ethiopia, Somalia and Sudan, mass starvation causes thousands of deaths due to PCM. Children are more susceptible to PCM than adults. The consequence of PCM are characterized by marked edema, hyperkeratotic or excoriated skin lesions, hepatomegaly, hypoalbuminemia, and anemia (20, 107). Furthermore, PCM can induce increased chromosomal damage. El-Ghazali *et al.* compared whole blood lymphocytic cultures between healthy and PCM infants and found

that the frequency of chromosomal damage in PCM children was nine times greater than that of healthy children (46). Schlesinger *et al.* reported that marasmic infants produce only 26% of the level of interferon that is detected in healthy infants (111). Since interferon, a glycoprotein which is released by leukocytes and suppresses viral replication, plays a critical role in the prevention of viral infections, marasmic infants suffer from more severe infections and have a higher mortality rate from viral infections compared to healthy infants (62, 111). In severe cases of PCM, there is marked atrophy of the thymus, which may result in immune suppression (72, 133). Smythe *et al.* reported involution of the thymus and smaller lymph nodes, tonsils, and spleens in children with PCM (115). Consequently, T cell mediated immune responses are depressed. This was demonstrated by the suppression of delayed cutaneous hypersensitivity reactions, decreased number of rosette-forming T cells, and decreased lymphocyte mitogenesis in response to PHA (1, 23, 24, 30, 45,52, 112, 115).

The effect of PCM on B cell mediated immune response has also been examined. In these studies, malnourished children had no differences or slightly elevated levels of serum immunoglobulins (Igs) in comparison to well-nourished children (3, 23, 76, 89). However, it was also reported that severe malnurition in infants profoundly suppressed immunoglobulin levels (47). It was suggested that the frequent gastrointestinal infections observed in malnourished children was responsible for the elevated levels of serum Igs. Immunization with a variety of antigens has been shown to evoke normal antibody responses in malnourished individuals (135). But in the case of a typhoid vaccination, the immune response of malnourished children to salmonella-specific antigens was lower than that of healthy children (23, 120). The reason for the reduced Ig response may be

explained by depressed cell-mediated immunity and T lymphocyte function in PCM which may imply that T lymphocytes could not adequately control the B-lymphocyte proliferation (120). Malnutrition also decreases the production of other serum proteins including the complement system (108, 109). Therefore, malnourished children are very vulnerable to bacterial, viral and parasitic infections. Moreover, Good and associates observed the marked similarities of symptoms between PCM and zinc deficiency children. This could be explained by the reduced zinc content in the serum of the PCM children. Therefore a large portion of impaired immune responses in PCM children may be attributed to dietary zinc deficiency of the children.

Trace Elements

It is estimated that about 45 to 50 dietary nutrients are required for health. They include nine amino acids, one or possibly two fatty acids, thirteen vitamins and a large number of inorganic elements. At least 14 inorganic elements are known to be essential for life and health. These are iron, copper, cobalt, iodine, zinc, selenium, manganese, molybdenum, chromium, fluoride, silicon, nickel, tin, and vanadium. They carry out a major role in many oxidation-reduction reactions and are an essential part of many enzymes, i.e., metalloenzymes. It is unusual to observe a dietary lack of these elements in normal people but in cases of marginal dietary intake compounded by metabolic interferences, impaired absorption or competition between metal ions may induce trace element deficiencies (107). These trace elements are required in an optimum concentration for proper functioning of the organism. Deficiency will result in suboptimal function, disease, and probably death. Similarly, excessive dietary

intake will lead to toxicity and may lead to a fatal outcome. Trace elements do function as catalytic or structural components of larger molecules like hormones and enzymes. Trace elements, in addition to other functions, also have been shown to regulate immune responses (25-27).

Zinc Metabolism

In 1869, Raulin was the first to discover that zinc was essential for the growth of *Aspergillus niger* (103). From that time, many investigators have described the essentiality of zinc for plant and animal growth. In swine, Kernkamp and Ferrin have described parakeratosis and Tucker and Salmon suggested that parakeratosis was due to a zinc deficiency (78, 127).

Zinc homeostasis is controlled primarily by the gastrointestinal tract, with the feces being the primary route of zinc excretion (32). Experiments utilizing ligated intestinal loops suggest that the duodenum is the major site of zinc absorption in rats (37). A substantial portion of zinc is found in the cytosol of the intestinal epithelial cell. In humans, approximately 28% of zinc reside in bone, 62% in muscles, 1.8% in the liver, and 0.1% in the plasma pool (98). With regards to the plasma zinc concentration, approximately 15% is tightly bound to α-macroglobulin, a small amount to amino acids, and the remainder is loosely bound to albumin (58). Albumin, a protein in plasma, serves as a carrier of newly absorbed dietary zinc and this albumin-zinc complex solely represents the readily exchangeable body zinc pool (58). During zinc deficiency, the extracellular zinc concentration drops precipitously while the other tissue zinc levels remain normal. For example, plasma zinc concentrations in rat can decrease by $\geq$50% within 24 h after the introduction of a zinc-deficient diet (40).

Zinc Malabsorption Syndromes

In 1961, Prasad *et al.* reported zinc deficiency in man. They reported severe growth retardation, anemia, hypogonadism, hepatomegaly, rough and dry skin and mental lethargy in a 21 year old Iranian (99). In 1963, they confirmed that zinc deficiency contributed to dwarfism and hypogonadism in Egypt. Because the habit of geophagia is common in middle Asia, they postulated zinc may form insoluble complexes with phosphate in soil and was not absorbed easily by the body (99, 100). Moreover, O'Dell and Savage observed that phytate (insoluble hexaphosphate), which is found in cereal grains, markedly impaired the absorption of zinc (93).

In 1972, Hambidge *et al.* reported zinc deficiency in American children from middle and upper income families (65). The children had symptoms of nutritional zinc deficiency such as growth retardation, poor appetite, and impaired taste acuity. After zinc supplementation, these symptoms were alleviated. They continued to study zinc deficiency in children from low-income families. The occurrence of zinc deficiency was more prevalent in children from low-income families than from middle or high income families. From these results, they suggest that zinc deficiency is correlated with food source rather than genetics (64). However, in 1973, Barnes and Moynahan discovered that acrodermatitis enteropathica (AE), an autosomal recessive inherited disorder, was closely related with zinc malabsorption. The symptoms of this disease are growth retardation, skin lesions, behavioral disturbances such as photophobia, malabsorption, diarrhea, and recurrent infections. After they detected low levels of serum zinc, they treated the patient with an oral administration of zinc sulfate which alleviated all symptoms (6, 19). Patients with gastrointestinal diseases,

ulcerative colitis (UC) and Crohn's disease (CD), also reportedly suffer from zinc deficiency symptoms. Inflammatory bowel disease (IBD) patients suffer from chronic inflammation of all layers of the bowel walls and this inflammation impedes absorption of nutrients and induces malnutrition. A portion of young IBD patients showed growth retardation, reduced weight gain, slowed skeletal maturation, and delayed sexual maturation (68, 92). Like AE, the supplementation of IBD patients with zinc abolished the symptoms. Even in adult, it was observed that zinc deficiency induces decreased semen volume and serum testosterone concentration in male (69).

Two genetic diseases associated with zinc metabolism have been reported in animals. In Holstein-Fresian cattle, lethal A-46, was studied by Flagstad in 1978. In calves with lethal A-46, skin lesions, involution of the thymus and immunological dysfunctions were observed. This genetic disorder was controlled in calves by a homozyous autosomal recessive gene and could be prevented by zinc treatment (55). In mice, Piletz and Ganshow reported lethal milk mutation in C57BL/6J (B/6) strain mice. Milk from dams expressing the recessive trait contained a significantly low level of zinc (97).

Zinc and Metalloenzymes

In 1940, it was first demonstrated that zinc was an integral and essential part for the normal function of carbonic anhydrase, a vital metalloenzyme (77). Zinc is also involved in nucleic acid and protein metabolism because key enzymes required for nucleic acid synthesis and degradation are zinc dependent (77). Therefore, zinc is an essential part in the process of cell differentiation and

replication. Currently, it is estimated that more than 200 enzymes need zinc as an important part of their activities (66).

During the prenatal and postnatal period, zinc is very critical for the normal growth of young animals. In pregnant rats, maternal dietary zinc deficiency severely impaired fetal growth and induced abnormalities, especially, in the central nervous system (CNS) (41, 70, 71). Many investigators insisted that thymidine kinase, a zinc dependent enzyme which controls mitotic activity, is responsible for congenital defects (42, 101). Using ^{3}H-thymidine incorporation technique, Swenerton *et al.* demonstrated the depression of nucleic acid synthesis in 12-day-old embryos of zinc deficient rats, especially in neural cells (121). Moreover, Dreosti *et al.* found reduced activity of L-glutamic acid dehydrogenase in zinc deficient neonates (43). The defect of the enzyme could increase concentration of glutamic acid, a putative neurotransmitter in the mossy fiber pathway, in the glutameric nerve terminals (36). Consequently, nerve impulse could be impeded by this enzymatic defect and affected animals showed behavioral abnormalities (43).

In humans, the first case of congenital malformation related with zinc deficiency was described by Hambidge *et al.* (67). They reported that from the seven pregnancies of mothers with AE, one was terminated in spontaneous abortion and two had congenital malformations, one with anencephalus and the other with achondroplasia (67). Similar inborn error incidents related with zinc deficiency were reported by several investigators (10, 21, 75, 84).

In regard to immunological development, Beach *et al.* demonstrated that the effect of zinc deficiency during the prenatal period manifested itself through three generations (8). When mice are deprived of zinc during the suckling period, their

ability to generate an effective immune response was also markedly compromised (9).

Zinc and Biomembranes

Even though a great portion of the defects associated with zinc deficiency can be ascribed to the metalloenzymatic dysfunctions, Bettger and O'Dell hypothesized that zinc exerts many vital roles on biomembrane structure and physiology. Zinc has a high affinity for proteins and lipoproteins located in cellular membranes and many authors reported the high concentration of zinc in the membrane fraction of a variety of organs (12). Zinc has been shown to maintain cell membrane integrity by several mechanisms. Bashford *et al.* reported that zinc protects red blood cells from hemolytic agents (7). Zinc is also known to protect membranes from peroxidative damages and has the potential to keep enzymes on the membrane surface (12). Zinc has been also shown to inhibit many calcium-mediated responses such as histamine release from stimulated mast cell, granulocyte chemotaxis and phagocytosis, and platelet contraction and aggregation (16). In sickle cell anemia, Eaton *et al.* found that sickle cell membrane damage is due to the increased concentration of intracellular calcium and zinc prevents the calcium induced shrinkage (44).

Snyder and Walker showed that zinc chloride supplementation significantly decreased mortality of mice injected with *Salmonella typhosa* lipopoly-saccharide (LPS). They hypothesized that zinc may protect mice from endotoxemia by stabilizing lysosomal membranes, which may prevent the release of degradative enzymes and other deleterious factors (116). Later, Sobocinski *et al.* demonstrated that zinc chloride supplementation ranging from

0.4 to 2 mg/100 g of body weight provided 80 to 100 % survival following challenge with *S. typhimurium* endotoxin in rats. Using ornithine carbamoyltransferase activity, they demonstrated the ability of zinc chloride to enhance the survival of hepatocytes following treatment with endotoxin (118).

Zinc and Immune Response

Zinc is also known as an essential element for the immune system. The primary influence of zinc deficiency is on various T cell functions. Flynn *et al.* has suggested that zinc deficiency may affect T cell function by depressing proliferation, interfering with antigen presentation by accessory cells, or inducing loss of cell function (57). Tanaka *et al.* observed that zinc enhanced the proliferation of T cells in response to IL-2, increased production of IL-2 by T cells and induced the expression of high affinity receptors for IL-2 on lymphocytes (123). Hambrige *et al.* suggested three hypotheses about how zinc deficiency deteriorate normal T cell functions (66). First, depression of activity of a zinc containing DNA polymerase, terminal deoxyribonucleotidyl-transferase, which is essential to the normal development of the thymus and immature thymocytes. Therefore, T cell maturation would be arrested (61, 88). Second, alteration of thymic epithelial function and impairment of thymic hormone production. Prasad reported reduced activity of thymulin, a thymus-specific hormone formery called facteur thymique serique (FTS), in the serum of mildly zinc deficient human subjects (98). Thymulin binds to receptors on T cells, induces the expression of several T cell markers, promotes T cell function including allogenic cytotoxicity and suppressor function, and enhances T cell differentiation (73, 98, 102). Third, increased incidence of programmed cell death of thymocytes. Cohen and Duke

reported that dexamethasone and corticosterone induce apoptosis (programmed cell death) of mice thymocytes by activation of an endonuclease. The endonuclease requires calcium or magnesium ions for activation but zinc suppresses its activation by competing with those ions, thus, protecting the thymocytes (28). In moderately zinc-deficient weanling rats, significantly reduced blastogenic responses to various mitogens were observed (63).

In *in vitro* assays, zinc deprivation of culture medium suppressed the release of IL-1 in allogeneic mixed lymphocyte cultures (57). In contrast, zinc supplementation of the medium enhanced the activity of IL-1 stimulated thymocytes (134). The modulation of IL-1 release and activity on thymocytes may be the mechanism for decreased antibody formation and decreased cell-mediated cytotoxicity observed during dietary zinc deficiency (81).

Neutrophils from moderately zinc deficient rhesus monkeys show significantly decreased chemotaxis to FMLP (129). In humans, Busino and associates reported that an infant which suffered from acrodermatitis had severe neutrophil chemotaxis defect but other neutrophil functions such as random motility, phagocytosis, and bactericidal activity were normal(19). In the cytotoxic T cell proliferation assay, 200 μM zinc allowed maximal growth of the cells but remained nonfunctional until zinc was removed from the media. This data implied that zinc enhanced clonal expansion of the cytotoxic T cells but suppressed the exertion of the function in some way. Zinc similarly inhibits natural killer cell activity (53). Zinc has been known to have an high affinity for free sulfhydryl group. Reardon and Lucas explained that zinc inhibits the effector function of the cytotoxic T cell by interacting with and blocking cell surface sulfhydryl groups which are required for the initiation of cell lysis and by

inhibiting cell surface protease activity which is necessary during lytic processes. They also explained the mechanism(s) by which zinc activate lymphocytes *in vitro* (80, 104). They postulated that it would be similar to a model for T lymphocyte activation by lectin mitogen and sulfhydryl-binding haptens. They explained that zinc may modify cell surface structures, class I and class II gene products, by cross linking structures with other cell-surface components to form a diversity of "altered self" structures. Therefore, T cell clones would recognize the "altered self" structures and respond to these foreign appearing cells (80, 104, 105, 130). Adult mice fed zinc deficient diets and subsequently percutaneously sensitized with dinitrofluorobenzene developed significantly lower DTH responses (49).

Although zinc deficiency is primarily known to affect cell mediated immune responses, antibody mediated immune responses were also affected by zinc deficiency. In antibody mediated immune responses, many investigators suggested that the duration of zinc deficiency and the age of an animal are the main factors associated with the onset of an impaired immune response (138). Prolonged zinc deficiency of adult animals or early imposition of zinc deficiency on young animals resulted in impairment of B cell mitogenesis to dextran (138). In responses to other antigens, whether T cell dependent or independent, zinc deprived animals produced smaller number of plaque forming cells (PFC) per spleen than animals fed adequate amounts of zinc (50). Many researchers suggested that primary antibody response is not independent of zinc status but is less sensitive to zinc deficiency than T cell responses. However, the secondary immune response is more reduced than the primary response in zinc deprived animals. In their experiment, DePasquale-Jardieu and Fraker

demonstrated that mice which were fed a zinc deficient diet for 4 weeks after priming with sheep red blood cell (SRBC) produced only 43% as many PFC per spleen as mice fed an adequate amounts of zinc (39). Furthermore, nutritional repletion restored only a portion of immunological memory cells (39). Because secondary immune response is exerted mostly by IgG, T cell helper function is needed to promote the switch of IgM to IgG. Therefore, secondary immune response is more vulnerable than primary immune response during zinc deficiency (51, 86).

Beach *et al.* showed that moderate zinc deficiency from day 7 of gestation until birth severely depressed immune functions of the newborn through 6 months of age. Even though all the zinc deficient newborns were fed normal diets, the depression of immune responses continued to the second and third filial generation with reduced effect (8).

Interactions between Zinc and Other Metals

The inhibitory effect of 10 μM cadmium on T cell proliferation was almost completely prevented by addition of 30 μM zinc to the culture medium. As intracellular cadmium content and a cadmium induced metallothionein level were not changed by zinc addition, these observations strongly suggest that cadmium inhibits some zinc dependent processes required for T cell proliferation (94). Eleven patients with newly diagnosed Wilson's disease were treated with zinc acetate as their anticopper therapy (17).

Macrophage and Immune Response

In vitro thymocytes and splenocytes culture in standard medium were not activated by any concentration of zinc from 10 to 400 μM. However, addition of macrophage culture supernatant fluid or cultures containing a monolayer of resident peritoneal macrophages restored the lymphocyte responses to zinc (104, 105). James *et al.* revealed that the observed depression in T cell proliferation in zinc deficient mice was indirect and due to a primary defect in the macrophages (74). When purified T cells from zinc deficient mice were cultured with macrophages from pair-fed control mice, they proliferated similar to control levels (74). Increased numbers of macrophages also overcome the impairment. Therefore, they postulated that the observed depression in T cell proliferation in the presence of zinc deficient macrophages was due to their insufficient activating activity rather than their increased suppressive activity (74). Adult human leukocytes cultured in a complete culture medium which containing 10% fecal bovine serum (FBS) secreted between 375 and 700 pg/ml of TNF. At 0.25 mM zinc, the leukocytes released the highest amount of TNF, but zinc concentrations above 1.0 mM were toxic to the cells (114). The amount of endotoxin in RPMI-1640 medium alone is estimated less than 6 pg/ml. This amount of endotoxin failed to induce cytokine secretion from cultured macrophages, however, the addition of LPS at concentrations above 0.01 μg/ml stimulated cytokine secretion in a concentration dependent manner. It was observed that combining a substimulatory concentration of 0.01 μg/ml LPS with zinc resulted in a synergistic stimulation of leukocyte TNF secretion (114). In case of zinc content, it was estimated that RPMI-1640 medium alone containing <0.5 μg/ml zinc and supplementation of 10% FCS increased the amount of zinc

to 30 μg/dl but still less than that observed in the plasma of zinc deficient mice (48 μg/dl). The restoration of lymphocyte response to zinc by the addition of macrophages may be due to the secretion of monokines. Thymocyte activation by zinc required the presence of 2-ME and LPS in concentrations as low as 1.0 ng/ml which may result in IL-1 secretion (74, 104, 105, 114).

The enhancing effects of 2-ME may be related to its reducing activity and to the high affinity of zinc for free sulfhydryl groups. The reduction of disulfide bridges by 2-ME, forming free thiols on the surface of thymocytes, may be necessary for zinc binding and for cell activation. Zinc stimulated IL-1β release in a concentration dependent manner with peak secretion occurring at 0.12 mM which was lower than that observed for TNF. Interleukin-6 secretion unlike TNF and IL-1β was unaffected by zinc concentration (74, 104, 105, 114).

Cytokines and Acute-Phase Reaction

Bacterial, viral, and parasitic infections, as well as trauma and many forms of neoplastic disease, elicit a well-characterized series of changes in host metabolism. These phenomena are called acute phase responses. This acute phase response is mediated by several hormones and cytokines such as IL-1, IL-6, and TNF-α (60, 82, 113, 131, 132).

Tumor necrosis factor is responsible for metabolic abnormalities frequently accompanying malignant neoplasms. At the cellular level, TNF has been shown to have pleomorphic effects on a variety of cells and this ability places this cytokine in a pivotal role in modulating acute and chronic inflammatory states (91, 119).

Endotoxin has been shown to be a potent agent capable of eliciting TNF production. Yet the mechanism that regulates TNF expression at the cellular and molecular levels has not been fully elucidated. Strieter *et al.* demonstrated that pentoxifylline, a methylxanthine, is able to suppress LPS induced macrophage derived TNF at the level of transcription and translation. Furthermore, methylxanthines and dibutyryl cAMP have similar effects on TNF expression. These data support the hypothesis that the suppressive mechanism is mediated via the generation of intracellular cAMP (91, 119).

Although blood monocytes and tissue macrophages appear to be the principal sources for TNF, recent studies suggest that natural killer cells, cytotoxic T cells, and bone-marrow-derived mast cells are capable of synthesizing TNF (124). It is now generally recognized that many putative beneficial responses of TNF are directed at improving the antimicrobial actions of leukocyte populations (91). In a study of murine TNF protection, mice were injected with antiserum specific for murine TNF. Injection of this serum into mice infected with the moderately mouse virulent *Salmonella typhimurium* strain M525 caused exacerbation of disease (124). Some biological actions previously attributed to IL-1 have now been ascribed to TNF. For example, TNF has been shown to be involved in neutrophil degranulation, superoxide production, and lysozyme release (91).

Experimental TNF injection induced shock, organ failure, and death (126). Goldberg and colleagues reported that TNF induced shock is completely alleviated by prostaglandin synthesis inhibitors (79). Since TNF has been known to be a potent inducer of prostanoid synthesis, injected TNF may mediate the production of excessive prostaglandin E_2 (PGE_2) and thromboxane B_2, which

together lead to vasoconstriction and neutrophil infiltration. However, the concomitant synthesis of PGE_2 is an important component in this system and may serve as an autocoid for transcriptional and post-transcriptional regulation of TNF via the second messenger cAMP (4, 5, 38, 83).

Prior treatment of macrophages with dexamethasone, an analogue of glucocorticoid, suppresses TNF production in response to endotoxin at the level of both transcriptional and posttranscriptional regulation similar to pentoxifylline (14, 91, 119). These data suggest the existance of a negative-feedback mechanism of TNF-induced corticosterone on TNF production (131). Gamma-interferon (γ-IFN) has been shown to augment macrophage synthesis of TNF. Although not an direct inducer of TNF synthesis, γ-IFN is thought to increase endotoxin induced TNF secretion by suppressing the synthesis of short lived repressors of TNF transcription (29). Warren *et al.* demonstrated that rats given TNF by intraperitoneal injection produced dose- and time- related increases in hepatic amino acid uptake, and enhanced amino acid release from skeletal muscle with a predominance in the efflux of the gluconeogenic amino acids, alanine and glutamine. Glucocorticoid released by TNF stimulation may contribute to this protein catabolism (131). TNF also induced a rise in C-reactive protein and a reduction in serum zinc (85). In human beings some acute phase proteins are subdivided into 3 groups according to their rate of change in the plasma: 1) the concentration may increase by about 50%, eg, ceruloplasmin and C_3, 2) it may increase 200 to 300%, eg, α1-acid glycoprotein, α1-antitrypsin, α1-antichymotrypsin, haptoglobin, and fibrinogen, and 3) a group containing those that may increase even up to 1000 fold. Among the latter are C-reactive protein (CRP) and SAA (15). Increases in serum amyloid A (SAA) concentrations have

been shown to coincided with decreasing serum zinc and iron concentrations; however, zinc and iron concentrations appeared to be restored when SAA concentration was still maximal (15). Serum amyloid A has also been found to possess immunoregulatory properties. Serum amyloid A helps to increase the removal of unwanted materials, such as bacterial endotoxin which are transported by high-density lipoproteins (125).

Like TNF, endotoxin is an inducer of IL-1. Even though many regulatory activities overlap, IL-1 is distinctive from TNF by fact that it has lymphocyte proliferative activity (128). Cytokines not only influence cellular metabolism, but also affect the secretion of hormones. The existence of a complete regulatory loop between IL-1 and glucocorticoids is well characterized. Interleukin-1 increases the secretion of glucocorticoids and glucocorticoids depresses the release of IL-1 from macrophages in a negative-feedback system (117, 132). During infections, IL-1 and TNF induce hypozincemia which may contribute to immunoregulation complementary to the increased release of glucocorticoids. In APR, the removal of iron and zinc from circulation to the liver is well demonstrated. Iron is known to be removed by lactoferrin (LF), a granulocyte released carrier molecule (82). In case of hypozincemia, metallothionein (MT) is a putative carrier molecule which removes zinc from circulation to the liver. Since TNF and IL-1 are potential inducers of IL-6, hypozincemia in APR may be mediated by IL-6 induced MT. In vitro culture of hepatocytes with IL-6 demonstrated the concentration- and time- dependent increase of MT-1 and -2 mRNA and MT protein (33, 34, 122, 137). Metallothionein is also induced by glucocorticoids and cadmium and is increased during periods of starvation, stress, and liver regeneration (35).

The addition of IL-1 to cultures of chicken skeletal muscle cells induced protein degradation about 24% greater than control cultures but did not affect protein synthesis (82). Enhanced skeletal muscle catabolism has also been observed in IL-1 injected rats (136). Goldberg and co-workers suggest that IL-1 increases the rate of protein degradation by a mechanism mediated by PGE_2. Elevated PGE_2 is thought to increase intracellular Ca^{2+} levels which in turn induces lysosomal protein release. Accelerated muscle proteolysis induced by crude IL-1 is neither diminished by the presence of insulin, nor is it synergestic with other catabolic hormones such as corticosteroid or glucagon (59). Several nutrients regulate the release of IL-1 from monocytes in nondeficient states. Since prostaglandins inhibit IL-1 release, it was hypothesized that ω3 fatty acids inhibited IL-1 release by affecting the production of arachidonic acid metabolites (48).

Interleukin-6 is a major cytokine mediator of MT gene expression and zinc metabolism in hepatocytes (113). The reason for acute phase hepatic MT induction and zinc accumulation is not yet clear. One potential benefit of moving zinc out of plasma and into organs such as the liver is to enhance zinc's availability to these tissues. Based on zinc's role in stabilizing membrane and MT's purpoted role as an oxygen radical scavenger, it has been proposed that zinc and MT may play important intracellular roles as antioxidents by protecting hepatocytes and other cells during infection when host-generated cytotoxic oxygen species are produced in large quantities. In several experiments, treatments that increased hepatocyte MT and zinc reversed the deleterious effects of CCl_4 on hepatocyte viability. These results are consistent with a functional role of zinc and MT in membrane stabilization (12, 31).

Metallothionein may serve to provide zinc to metalloenzymes and to "zinc finger" motifs of DNA or may act to buffer intracellular zinc concentrations for these or a variety of other functions. Also, MT may play a cytoprotective role as a scavenger of hydroxyl or oxygen radicals or as a donor of zinc for stabilization of membranes (12, 35, 95).

The consequences of acute-phase responses are anorexia, loss of lean body mass, fever, gluconeogenesis, hepatic secretory protein synthesis, and trace element redistribution (60, 114, 119). Characteristic changes in trace element metabolism are an integral part of the acute phase response. These changes are usually reflected in increased serum copper and decreased serum iron and zinc levels. Decreased serum Zn levels apparently result from the synthesis of MT in liver and other tissues (85, 128, 132). It appeared that TNF plays a principal role in the down regulation of the albumin gene as well as reducing the rate of albumin mRNA translation (31).

The purpose of this study was to determine whether the production of IL-1, IL-6, or TNF by murine peritoneal macrophage was affected by the onset of zinc deficiency.

THE EFFECT OF ZINC DEFICIENCY ON THE PRODUCTION OF INFLAMMATORY CYTOKINES FROM MURINE MACROPHAGES

by

Haa-Yung Lee

INTRODUCTION

Generally, malnutrition and select trace mineral deficiencies are recognized as one of the causes of an impaired immune response. Furthermore, nutritional deficiency can initiate and make worse several disease states (10).

The trace element zinc (Zn), has been shown to be an essential nutrient for normal physiological function for more than one century. Even though the frequency and severity of Zn deficiency in human populations is an issue of considerable debate, there are several high risk groups. These groups include infants, adolescents, women of reproductive ages, and the elderly (8, 14, 18, 28).

The signs and symptoms of zinc deficiency vary with the length and degree of the deficiency and the age and species of the affected animal. Characteristic physiological and biochemical changes associated with zinc deficiency are anorexia, growth retardation, alopecia and skin lesions, reproductive abnormality, skeletal defects, impaired wound healing, decreased immunocompetence, decreased concentration of plasma zinc, decreased enzyme activity in plasma and tissues, and increased lipid peroxidation (2).

In humans, many investigators have demonstrated that zinc deficiency contributes to the severity and pathogenesis of several diseases. A representative of these diseases is inflammatory bowel disease (IBD) which includes ulcerative colitis and Crohn's disease. The etiology of IBD remains unknown, with several theories postulating a genetic, infectious, psychosomatic, or immunologic origin (1, 8, 18).

Recent studies indicate that zinc is needed for development of immune responses (3, 19, 20, 27). It has been known that zinc deficiency causes involution of thymus and spleen but does not affect vital organs such as brain, liver, or heart (26). Reduced numbers of circulating white blood cells, i.e., leukopenia, are associated with a small thymus and spleen. A reduction in the number of lymphocytes, especially T lymphocytes, mainly contributes to the leukopenia associated with zinc deficiency (2). However, the numbers of peripheral blood neutrophils and monocytes of zinc deficient animals are within normal range (10).

One consequence of zinc deficiency is adrenal hypertrophy and an increase in circulating glucocorticoids which accelerates thymic atrophy (18). Other reports also suggest that zinc deficiency may alter thymic epithelial function and impair thymic hormone, thymulin, production, which in turn would inhibit T cell maturation in the thymus and the periphery. Consequently, induction of several T cell markers, and promotion of T cell functions including allogenic cytotoxicity, suppressor functions, interleukin-1 (IL-1) production, and T cell differentiation are restricted (9, 15, 17). Among the T cell subsets, T helper cells are the most vulnerable to zinc deficiency and it is testified by the decreased ratio of $T4^+/T8^+$ lymphocytes (18). These alteration in the immune system contribute to the immune dysfunction observed in zinc deficient animals (4, 12).

When pathogens or inflammatory agents invade the body, the host responds not only by mounting specific and nonspecific immune responses against the causal agent but also by initiating a well-characterized series of metabolic adjustments which is often referred to as the acute phase response (APR). This response can cause decreased food intake, increased resting energy

expenditure, gluconeogenesis, glucose oxidation, hepatic synthesis of fatty acids and acute phase proteins, decreased fatty acid uptake by adipocytes, and altered distribution of zinc, iron, and copper. This response is mediated by several hormones and host products released from stimulated leukocytes and these products are referred to as cytokines. The three cytokines that are best known as regulators of the acute-phase response are IL-1, tumor necrosis factor-α (TNF-α), and IL-6 (11, 22).

Zinc ion has also been shown to protect mice against lethal endotoxin shock (24). The zinc ion appears to mediate the stabilization of the cell membrane, so that lysosomal mediators, especially proteases, can not be released. Zinc has been shown to protect a variety of membrane systems against peroxidative damage induced by carbon tetrachloride or high oxygen tension (24, 25).

The impact of zinc deficiency on the induction of an inflammatory response and cytokine production is not clearly understood. There are several questions which still remained unresolved: 1) whether inflammatory bowel disease induces zinc deficiency or zinc deficiency provokes inflammatory bowel disease; 2) whether inflammation in zinc deficient animals was worsened by enhanced production of cytokines or by reduced resistance of biomembrane; 3) whether reduced concentration of cytokines in the serum of zinc deficient animals was due to the depressed production of cytokines on a cellular basis or to the decreased number of cells capable of producing cytokines. The purpose of this study was to determine whether the production of IL-6, or TNF by murine peritoneal macrophage was affected by the onset of zinc deficiency.

MATERIALS AND METHODS

Experimental animals

Five to six week old BALB/cByJ mice were used. Mice were originally purchased from Jackson Laboratories, Bar Harbor, ME. All mice for this work were obtained from breeding colonies maintained in the Laboratory Animal Facility of the College of Veterinary Medicine, Iowa State University. All breeding colonies were maintained on Mouse Lab Chow #5010 (Purina Mills, inc., St. Louis, MO). For the control diet, mice were fed Mouse Lab Chow which will be referred to herein as the "Normal" diet. In these experiments, weanling mice were fed normal diet for 1 or 2 weeks, then divided into two groups designated as zinc deficient (Zn^-) and zinc sufficient (Zn^+) within gender groups and they were fed the respective diets for an additional 3 or 6 weeks. Zinc sufficient diet (Harlan Teklad, TD 85420, Madison, WI) contained 50 parts per million (ppm) zinc and zinc deficient diet (Harlan Teklad, TD 85419, Madison, WI) contained less than 1 ppm zinc. Both the zinc sufficient (TD 85420) and deficient (TD 85419) diets were nutritionally complete and only differed in the amount of zinc present in each of the diets.

Collection of peritoneal exudate cells

Peritoneal exudate cells (PECs) were collected according to the following protocol. Mice were injected i.p. with 2 ml of mixture containing 1.5% thioglycollate (Difco Laboratories, Detroit, MI) and proteose peptone (0.5 % solution, Difco Laboratories, Detroit, MI). Three days post-inoculation, mice

were sacrificed by cervical dislocation. Peritoneal macrophages were collected aseptically by flushing the peritoneal cavity twice with 7 ml pyrogen-free phosphate buffered saline (PF-PBS; 136.9 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.2) containing 10 units/ml heparin (Elkins-Sinn, Inc., Cherry Hill, NJ) and 1% fetal bovine serum (FBS, JR Scientific, Irvine, CA). After washing with PF-PBS one time, cells were washed twice with pyrogen-free minimal essential medium (MEM, Sigma Chemical Co., ST. Louis, MO) supplemented with 3.7% $NaHCO_3$, 25 units/ml penicillin, 25 μg/ml streptomycin, and 2 mM L-glutamine, and then resuspended in pyrogen-free MEM. Cells were counted and dispensed at 0.25 x 10^6 PECs/ml in each well of 12 well culture plates (Costar, Cambridge, MA) and treated with 0, 0.03, 0.1, 0.3, 1, or 3 μg/ml *Escherichia coli* K235 endotoxin (TCA extracted, Sigma Chemical Co., St. Louis, MO) respectively and incubated for 8 or 12 hr at 37°C in a 5% CO_2 humidified incubator. Supernatant fluids were harvested and after centrifugation (model TJ-6, Beckman, Palo Alto, CA) at 1000 rpm for 10 min, the cell-free supernatants were stored at -70°C.

Harvesting serum

Experimental mice were injected i.p. with 1 μg *Escherichia coli* K235 lipopolysaccharide (*E. coli* LPS, phenol/water purified, Sigma Chemical Co., St. Louis, MO) suspended in 1 ml pyrogen-free saline and control mice received an i.p. injection of pyrogen-free saline alone (1 ml). One and half hour later, blood samples were obtained by retro-orbital puncture. Serum was collected, centrifuged, and stored at -70°C.

TNF Bioassay

Murine L929 cells were maintained in L929 media (MEM supplemented with 10% horse serum (HS, Hyclone, Logan, Utah)). Cells were removed by scrapping, washed twice, and resuspended in L929 media at 0.25 x 10^6 cells/ml. One tenth ml of cells was added to each well of a 96 well microtiter plate (Costar, #3268, Cambridge, MA) and incubated overnight at 37°C in a 5% CO_2 incubator (model GCA, Precision, Bedford, MA). Samples were diluted in TNF media (L929 media plus 3 μg Actinomycin D/ml media (Sigma Chemical Co., St. Louis, MO)) and added to wells of L929 monolayers as a 1:1 dilution. The plates were incubated for an additional 18 hr. The cells were fixed with 50 μl of 10% phosphate buffered formalin (PBF) for 10 min and non-adherent cells were removed by washing with a saline solution. Residual monolayers were stained with a 100 μl of 0.2% crystal violet (#73136, Fisher Scientific Co., Fair Lama, NJ)-20% formalin solution/well for 20 min and excessive crystal violet was removed by washing 3 times with tap water. The plates were dryed then the remaining crystal violet was solubilized with 100 μl of absolute alcohol in PBS (1 vol : 1 vol) and quantitated by measuring optical density (O.D.) at 595 nm on an ELISA reader (model EL 301, Biotek Instruments, Winooski, VT).

IL-6 Bioassay

The B-9 subclone of an IL-6 dependent cell line (obtained from Dr. Mike Murtaugh, University of Minnesota) was maintained in RPMI-1640 media (Sigma Chemical Co., St. Louis, MO) supplemented with 3.7% $NaHCO_3$, 25 units/ml penicillin, 25 μg/ml streptomycin, 2 mM L-glutamine, 5% FBS, 50 μM 2-mercaptoethanol, and 3.3 x 10^3 u/ml rIL-6 (#HIL-6-D, Genzyme, Cambridge,

MA) for the normal growth of the cells. Cells were harvested from the tissue culture flasks (Costar, Cambridge, MA), washed 3 times with RPMI-1640 and counted. Cells were resuspended to 5 x 10^4 cells/ml in RPMI with 5% FBS and dispensed at 100 µl per well in 96 well culture plates. Supernatant samples were then diluted and added at 100 µl/well. The cells were cultured for 64 hr at 37°C in 5% CO_2 then pulsed with ^{3}H-thymidine. After 6 hr additional incubation, cells were harvested on glass-filter paper and the incorporation of ^{3}H-thymidine was measured by a liquid scintillation counter (model 1500, Packard, Downers glove, IL).

Alkaline phosphatase activity measurement

Sera of mice were diluted with ELISA media (#104-0, Sigma chemical Co., St. Louis, MO). For the standard, diluted alkaline phosphatase (#075-1806, Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) were used. The substrate was added and the O.D at 405 nm was measured at 0, 5, 15, 30, and 60 min.

Statistical Evaluation

Student's t-distribution was used to determine significance.

RESULTS

The body weight changes of three groups of mice which were fed normal, zinc deficient (ZD) or zinc sufficient (ZS) diets are represented in Figure 1, and corresponding alkaline phosphatase activities are expressed in Figure 2. In the weight gain comparison, mice fed the ZD diet gained the least amount of weight, regardless of gender. In contrast, mice pair-fed the ZS diet weighed approximately 80% of the weight determined for mice fed the normal diet *ad libitum*. In contrast, the ZD female mice gained about 50% less than normal female mice while male mice only gained 27% of the weight gained by male mice fed normal lab chow (Fig. 1).

Alkaline phosphatase has been used as one of the indicator enzymes of zinc deficiency. The serum alkaline phosphatase activity in the serum of ZD mice was less than that determined for ZS and control mice (Fig. 2). The reduction in alkaline phosphatase activity was evidenced by the decreased slope obtained from the line generated by the alkaline phosphatase activity in the serum of the ZD mice (Fig. 2).

In the comparison of organ sizes among mice fed the three different diets, the mice fed the ZD diet had the smallest spleens and thymuses regardless of gender (Table 1). Between thymus and spleen, the involution rate of thymus was greater than that of spleen. One of the interesting results was that at the same age in the same diet group, usually female mice had bigger organs than those of males, in terms of both absolute and relative organ weight (Table 1). The range of organ sizes among the three diet groups was also bigger in female mice than

in male mice. The changes of organ weights from three weeks to six weeks were thought to be synchronous effects of both long-term dietary zinc deficiency and in part natural changes of the organs (Table 1).

The production of TNF from PECs was dependent upon the dose of *E. coli* endotoxin with 3 μg/ml as the dose which stimulated maximal activity of PECs. Compared to that of female mice, PECs from male mice demonstrated a larger difference in the production of TNF depending upon the diet. (Fig. 3). Peritoneal macrophages recovered from mice fed a ZS diet produced more TNF than PECs from mice fed a ZD diet (Fig. 3). When cultured for more than 8 hr, PECs from mice fed the ZD diet produced less TNF than PECs from ZS mice. This was even true for PECs recovered from the less sensitive female mice (Fig. 4). The time point production experiment, both diet groups produced comparable amounts of TNF from 1 hr to 8 hr post-treatment, however, after 8 hr incubation, the amount of TNF produced from PECs of ZD mice decreased rapidly compare to that produced by PEC from ZS animals. The maximal TNF production was reached at 4 hours post-treatment (Fig. 4).

The results obtained from the IL-6 bioassay were comparable with the TNF assay results. Both in male and female mice, PEC from ZS mice secreted more IL-6 than did the PEC from ZD mice (Fig. 5). The PEC recovered from ZD male mice, however, secreted very little IL-6 regardless of the endotoxin dose. In contrast, the IL-6 production from PEC obtained from female mice was closely dependent upon the dose of endotoxin. At a dose of 3 μg/ml endotoxin, ZD female mice produced as much IL-6 as their ZS counterparts but at a dose $\geq$ 0.1 μg/ml endotoxin, the production of IL-6 was not detectable. In contrast, there was

no significant difference in the amount of IL-6 produced by PEC from ZS mice over a wide dosage range of endotoxin (Fig. 5).

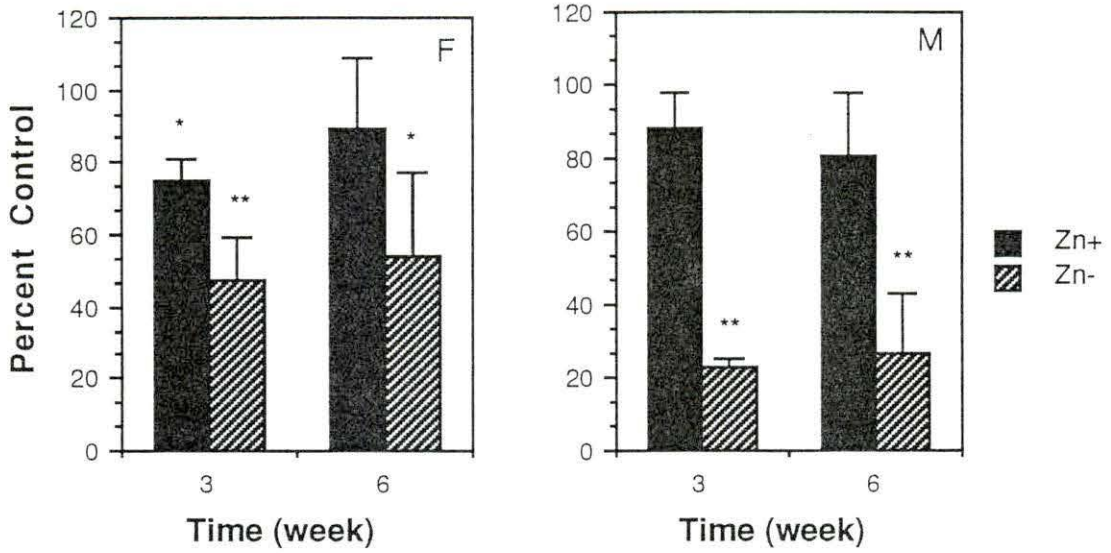

Figure 1. Percentage weight gain of mice which were fed normal diet ad libitum (Mouse Lab Chow), pair-fed the zinc sufficient diet (50 ppm, Zn+), or zinc deficient diet ad libitum (1 ppm, Zn-) for 3 or 6 weeks. Each value is expressed as the mean (n= 8 to 10) ± S.E.M. The percentage scores indicate relative weight gain in the treatment groups compared to normal weight gain. F refers to female and M refers to male. The body weights of the normal mice after 3 week diet treatment were 20.2 ± 1.6 and 22.7 ± 1.2 and after 6 week diet treatment were 21.8 ± 2.1 and 27.6 ± 1.8 in female and male mice respectively. p values are expressed compare to normal diet mice.
*, $p<0.05$
**, $p<0.005$

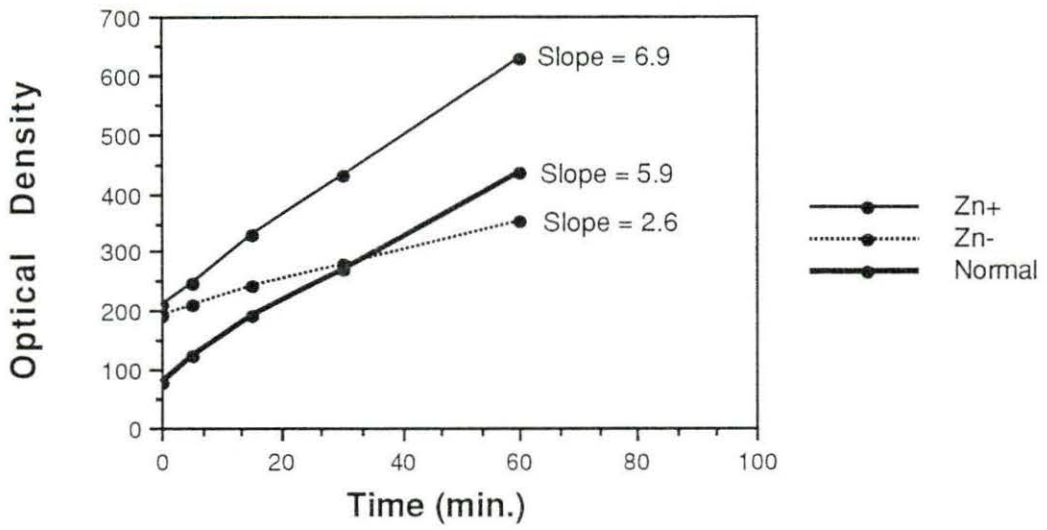

Figure 2. Kinetics of serum alkaline phosphatase activity of mice which were fed 3 different diets for 3 weeks. Activities of the enzyme were measured at 0, 5, 15, 30, and 60 min after adding substrate. Each point represents average of 5 individual measurements. Slopes are expressed by dividing the O.D difference of the enzymatic activity with time. Serum was collected from 5 mice in each group.

Table 1. Spleen and thymus weights of mice fed 3 different diets.

Treatment group[a]	3 Weeks Posttreatment[b]	
	Spleen[c] (mg)	Thymus[c] (mg)
Male		
Normal	N.D.[d]	40.8 ± 12.5
Zn^+	110.6 ± 22.3	35.3 ± 09.0 *
Zn^-	75.1 ± 18.6 #	22.1 ± 07.4 **
Female		
Normal	N.D.	78.2 ± 15.7
Zn^+	112.7 ± 14.8	55.5 ± 08.9 **
Zn^-	91.0 ± 19.3 #	34.9 ± 13.1 **
	6 Weeks Posttreatment	
	Spleen (mg)	Thymus (mg)
Male		
Normal	93.2 ± 04.4	40.0 ± 08.5
Zn^+	80.5 ± 05.0 *	45.8 ± 15.9
Zn^-	61.0 ± 13.3 **	17.8 ± 03.6 **
Female		
Normal	112.5 ± 08.3	57.8 ± 04.5
Zn^+	91.6 ± 14.0 **	37.6 ± 04.4 **
Zn^-	64.0 ± 18.2 **	25.0 ± 08.9 **

[a] Nor refers to mice fed the Purina lab chow (#5010), Zn^+ refers to mice fed the Teklad diet (TD 85420) containing 50 ppm zinc, and Zn^- refers to mice fed the Teklad diet (TD 85419) containing 1 ppm zinc.

[b] Organ weights were measured at both 3 weeks and 6 weeks after initiation of the treatment. Separate groups of mice were used for each time point.

[c] Measurements are expressed as the mean (n= 8-10) ± S.E.M.

[d] N.D.=not determined.

*, $p<0.05$ compare to normal values at the time point within gender.

**, $p<0.005$ compare to normal values at the time point within gender.

#, $p<0.05$ compare to zinc sufficient mice.

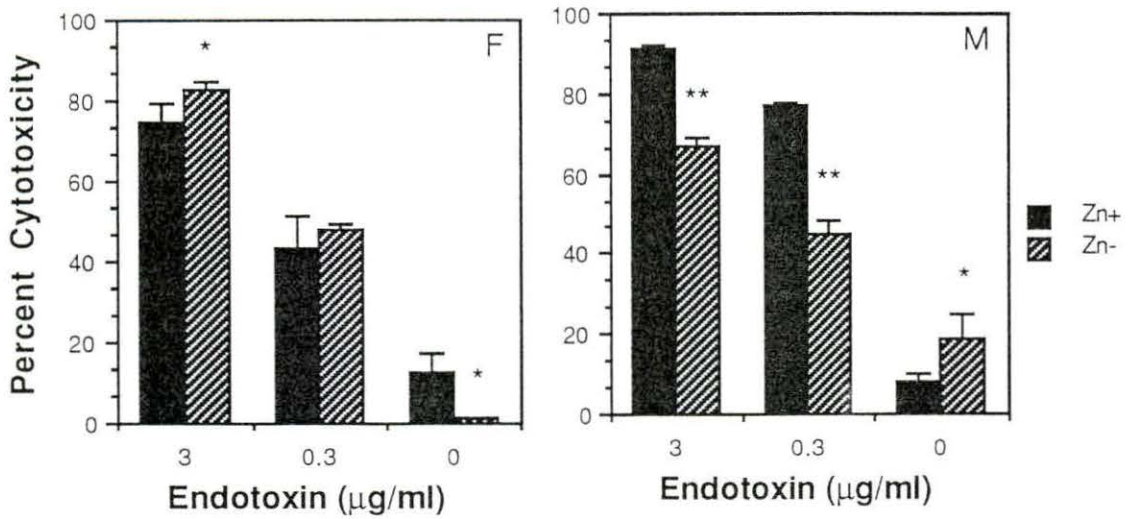

Figure 3. Tumor necrosis factor activity in the culture supernatant fluid of macrophages recovered from mice fed zinc sufficient or zinc deficient diets for 6 week. Harvested macrophages were cultured at 0.25 x 10^6 cells/ml concentration. Data is presented as the percentage cytotoxicity of L929 cells induced by individual culture supernatant fluids (1:20 dilution) from macrophages treated with the indicated amounts of *E. coli* K235 endotoxin for 8 hr. Each point is a mean ± S.E.M. of 6 measurements.

*, $p<0.05$, compared to zinc sufficient treatment value.

**, $p<0.005$, compared to zinc sufficient treatment value.

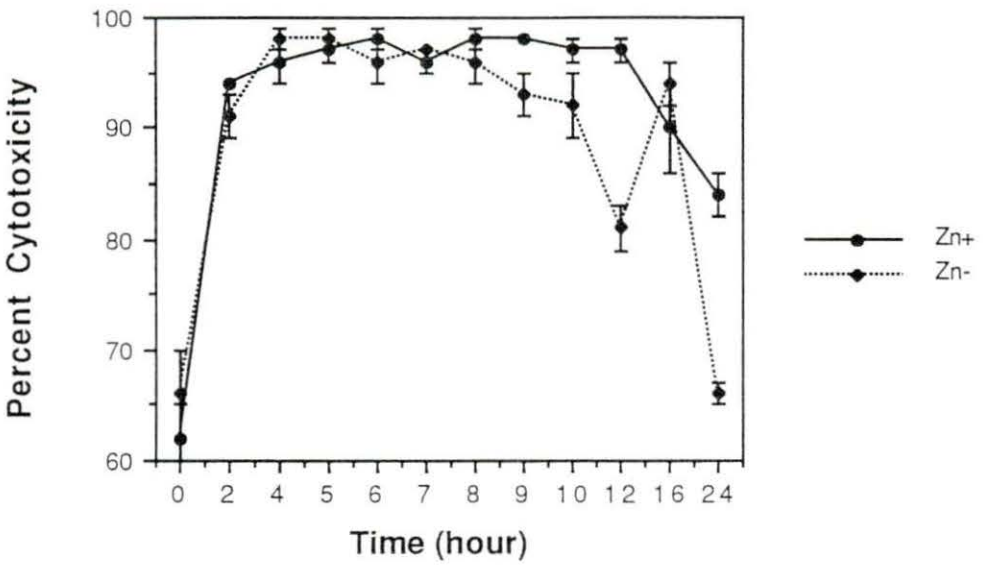

Figure 4. Comparison of tumor necrosis factor activity in the culture supernatant fluid of peritoneal macrophages recovered from female mice fed either a zinc sufficient or deficient diet. Supernatant fluid was collected at various times following initiation of the treatment (*E. coli* K235 endotoxin at 1 μg/ml). Data are presented as the mean ± S.E.M. of the percentage cytotoxicity of L929 cells.

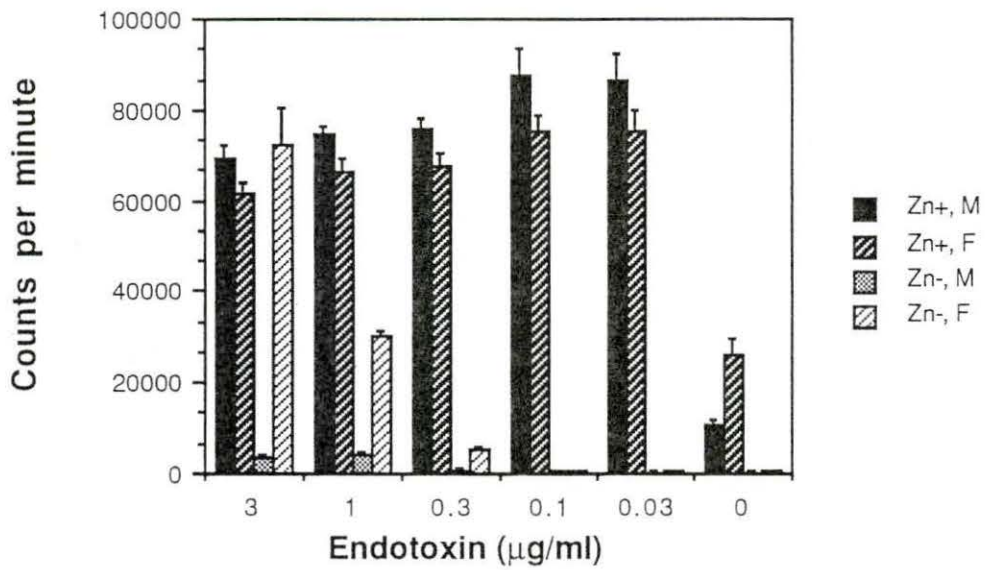

Figure 5. Production of interleukin-6 by murine peritoneal exudate cells after stimulation with *E. coli* K235 endotoxin. Both male (M) and female (F) mice were fed either zinc sufficient (Zn^+) or zinc deficient (Zn^-) diet for 6 weeks. Data are presented as the mean ± S.E.M. counts per minute of triplicate cultures as described in Materials and Methods.

DISCUSSION

Various effects of zinc deficiency have been described by many investigators. Of these effects, retarded growth or weight gain is readily demonstrable. In agreement with previous work (9, 15, 19, 29), we observed retarded weight gain of ZD mice (Fig. 1). This failure to grow can be explained by reduced appetite or food intake of mice fed a ZD diet (6). It has also been described that ZD animals have poor energy efficiency and high energy cost for the maintenance of normal physiology (7). However, reduced food intake can not be the only reason for the poor growth because, compared to pair-fed mice, ZD mice still have significantly lower weight gain in comparison to mice fed the normal lab chow (in females 88% and 54%; in males 81% and 27%, respectively) (Fig. 1). In this experiment, the discrepancy of weight gain between ZD male and female mice was also observed. During the 6 week trial period, ZD female mice gained approximately 50% of the weight gained by normal female mice, while ZD male mice only added about 27% of the weight gained by normal male mice (Fig. 1).

Zinc has been shown to be an essential part of many metalloenzymes (7). Alkaline phosphatase is one of the well-known metalloenzymes which contains four atoms of zinc per enzyme and is very sensitive to zinc deficiency. Therefore, alkaline phosphatase can be a useful indicator of zinc deficiency. Roth and Kirchgessner observed rapid decrease of serum alkaline phosphatase activity in rats (22). In their experiment, serum obtained from animals fed a ZD diet for 2 days had 75% of the alkaline phosphatase activity

detected in the serum from control animals, and serum collected after 4 days dietary depletion had a 50% reduction in activity. In our mouse model, the results were compatible with those of Roth and Kirchgessner. The activity of alkaline phosphatase in the serum of mice fed the ZD diet was lower than that of mice fed a normal or ZS diet (Fig. 2). The decreased serum alkaline phosphatase activity observed from mice fed the ZD diet could be explained by the low activity of the enzyme or a decreased amount of the enzyme (7). However it is more likely that a decrease in available zinc resulted in the decreased activity. The total serum zinc concentration was reduced in mice fed a ZD diet (data not shown).

Zinc deficiency also has been known to affect lymphoid organs, especially thymus and spleen. Both organs are rapidly involuted during periods of zinc deficieny (5, 7, 14). In comparison to normal mice, mice fed the ZD diet for 3 weeks showed greater differences in organ weights than mice fed the ZD diet for 6 weeks (Table 1). Involution of the thymus after the adolescent period is a well-studied phenomenon. This phenomenon can partially explain the greater difference of organ weight observed in mice at 3 weeks posttreatment in comparison to mice at 6 weeks. Therefore, the differences in organ size observed between mice at 6 weeks and 3 weeks may have occurred as a result of natural involution of the organ in combination with the effects of long-term zinc deficiency. Another interesting observation was the difference in organ sizes between male and female within a treatment group. Among all 3 diet groups, at both the 3 and 6 week time points, female mice had bigger organs than their male counterparts (Table 1). Previously, other investigators have also observed gender-related differences in the responses to zinc deficiency (7, 24),

but these observations are not universally accepted (7, 24). However, in this experiment, a clear gender difference was observed with respect to the size of the thymus and spleen, total weight gained, and mortality (data not shown).

Tumor necrosis factor-α or cachectin has been known as a pluripotent cytokine in inflammatory responses (12, 23). In animal models, i. p. injection of bacterial LPS, or endotoxin can elicit serum TNF production (16). Murine peritoneal exudate cells (PEC) treated with *E. coli* endotoxin in vitro will produce TNF. The amount of TNF produced by the endotoxin stimulation corresponds with the dose of endotoxin used with a maximal response obtained at a dose of 3 μg/ml (Fig 3). In the present experiment, PECs from ZS and ZD female mice did not show significant difference in the production of TNF. However, the differences in TNF production by PECs from male mice fed ZD or ZS diets were significant (Fig. 3). Even though female mice appeared to be less sensitive to the effects of zinc deficiency, PEC collected from female mice fed the ZD diet produced less TNF than control mice when the supernatant fluid was collected later than 8 hr after the initiation of the culture (Fig. 4). From this observation, it was postulated that the amount of available zinc in the serum of a animal is a limiting fact to regulate synthesis of cytokines. Since zinc is an essential element for numerous metalloenzymes, PEC from ZD mice may have depressed enzymatic activity after use up available zinc in the cell (8).

The production of IL-6 appeared to be more sensitive to nutritional zinc deficiency than was the production of TNF (Fig. 5). The production of IL-6 from PEC recovered from ZD female mice was only detected following treatment with ≤0.3 μg/ml endotoxin. Peritoneal exudate cells from ZD male mice did not produce detectable amounts of IL-6 when stimulated with endotoxin (Fig. 5).

However, PECs from mice fed the ZS diet secreted large amount of IL-6 even at the lowest dose of endotoxin tested. This result suggests that a change in the stimulatory threshold of PEC from ZD mice. To provoke a cytokine response from ZD animals, either an increase in the amount of endotoxin is required or other co-factors are required to augment the action of endotoxin. It is possible that the results observed in vitro will not be observed in vivo since many other cell types are present and may provide the necessary co-factors for the initiation of a cytokine response. In fact, there were no differences in the serum levels of TNF following i.p. injection of endotoxin, regardless of the dietary treatment (data not shown).

These results confirm the importance of zinc in host immune responses to outside pathogens via cytokine production. In addition, it was observed that female and male mice adjusted differently to dietary zinc deficiency. Female mice were less effected by zinc deficiency than male mice and the PEC recovered from female mice were more responsive to endotoxin than were PEC from male mice. These differences in cytokine production may be the consequence of the dietary zinc deficiency. The endocrine and physiological differences between male and female mice may contribute to the relative susceptibility to zinc deficiency. In conclusion, these data indicate that dietary zinc deficiency can alter the ability of PECs to produce inflammatory cytokines and that this effect is compounded by the gender of the animal.

REFERENCES

1. **Ainley C., J. Cason, B. M. Slavin, R. A. Wolstencroft, and R. P. H. Thompson.** 1991. The influence of zinc status and malnutition on immunological function in Crohn's Disease. Gastroenterology. **100:** 1616-1625.

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

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

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

5. **Good, R. A., A. West, N. K. Day, Z. W. Dong, and G. Fernandes.** 1982. Effects of undernutrition on host cell and organ function. Cancer Res. **42:**737S-743S.

6. **Hambidge, K. M., P. A.Walravens, R. M. Brown, J. Webster, S. White, M. Anthony, and M. L. Roth.** 1976. Zinc nutrition of preschool children in the Denver Head Start Program. Am. J. Clin. Nutr. **29:**734-738.

7. **Hambidge, K. M., C. E. Casey, and N. F. Krebs.** 1986. Zinc. In Trace Elements In Human And Animal Nutrition. W. Mertz, ed. Academic Press Inc. San Diego. pp1-137.

8. **Hambidge, K. M., C. E. Casey, and N. F. Krebs.** 1986. Zinc. In Trace Elements In Human And Animal Nutrition. W. Mertz, ed. Academic Press Inc. San Diego. pp1-137.

9. **Hendricks, K. M., and W. A. Walker.** 1988. Zinc deficiency in inflammatory bowel disease. Nutr. Review. **46:**401-408.

10. **Iwata, T., G. S. Incefy, T. Tanaka, G. Fernendes, C. J. Menendez-Botet, K. Pih, and R. A. Good.** 1979. Circulating thymic hormone levels in zinc deficiency. Cell. Immunol. **47:**100-105.

11. **Keen, C. L., and E. M. Gershwin.** 1990. Zinc deficiency and immune function. Annu. Rev. Nutr. **10:**415-31.

12. **Klasing, K. C.** 1988. Nutritional aspects of leukocytic cytokines. J. Nutr. **188:**1436-1446.

13. **Leucke, R. W., C. E. Simonel, and P. J. Fraker.** 1978. The effect of restricted diet intake on the antibody mediated response of the zinc deficient A/J mouse. J. Nutr. **108:**881- 887.

14. **McCaffrey, R., D. F. Smoler, and D. Baltimore.** 1973. Terminal deoxyribo-nucleotidyl transferace in a case of childhood lymphoblastic leukemia. Proc. Natl. Acad. Sci. USA. **70:**521-525.

15. **Moldawer. L. L., and S. F. Lowry.** 1988. Cachectin: Its impact on metabolism and nutritional status. Ann. Rev. Nutr. **8:**585-609.

16. **Nibbelink, S. K.** 1992. The role of tumor necrosis factor and secondary bacteria in the pathogenesis of swine dysentery. Ph.D. dissertation, Iowa state University.

17. **Prasad, A. S.** 1991. Discovery of human zinc deficiency and studies in an experimental human model. Am. J. Nutr. **53:**403-412.

18. **Prasad, A. S., S. Meftah, J. Abdallah, J. Kaplan, G. J. Brewer, J. F. Bach, and M. Dardenne.** 1988. Serum thymulin in human zinc deficiency. J. Clin. Invest. **82:**1202-1210.

19. **Prasad, A. S.** 1991. Discovery of human zinc deficiency and studies in an experimental human model. Am. J. Clin. Nutr. **53:**403-412.

20. **Reardon, C. L., and D. O. Lucas.** 1987. Heavy-metal mitogenesis: Thymocyte activation by Zn^{++} requires 2-mercaptoethanol and lipopolysaccharide as cofactors. Immunobiol. **174:**233-243.

21. **Reardon, C. L., and D. O. Lucas.** 1987. Heavy-metal mitogenesis: Zn^{++} and Hg^{++} induce cellular cytotoxicity and interferon production in murine T lymphocytes. Immobiol. **175:**455-469.

22. **Roth, H. P., and M. Kirchgessner.** 1980. Zn metalloenzyme activities. Wld Rev. Nutr. Diet. **34:**144-160.

23. **Scuderi, P.** 1990. Differential effects of copper and zinc on human periperal blood monocyte cytokine secretion. Cell. Immunol. **126:**391-405.

24. **Shapcott, D.** 1982. Hair and plasma in the diagnosis of zinc deficiency. In Clinical Applications of Recent Advances in Zinc Metabolism. A. S. Prasad, I. E. Dreosti, and B. S. Hetzel, eds. Alan R. Liss, inc. New York. pp121-139.

25. **Snyder, S. L., and R. I. Walker.** 1976. Inhibition of lethality in endotoxin-challenged mice treated zinc chloride. Infect. Immun. **13:**998-1000.

26. **Sobocinski, P. Z., M. C. Powanda, W. J. Canterbury, S. V. Machotka, R. I. Walker,and S.L. Snyder.** 1977. Role of zinc in the abatement of hepatocellular damage and mortality induced in endotoxemic rats. Infect. immun. **15:**950-957.

27. **Song, M. K.** 1987. Influence of dietary zinc content on sodium and potassium Metabolism in the rat. Mineral Electrolyte Mtab. **13:**178-182.

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

29. **Woodward, B.** 1991. Zinc, a pharmacologically potent essential nutrient: focus on immunity. Can. Med. Assoc. J. **145:**1469.

GENERAL SUMMARY

The effect of zinc deficiency on the ability of cytokine production by murine macrophage was studied. This paper showed a differential effect of zinc deficiency between male and female mice. Generally, ZD male mice suffered more severely than ZD female mice in terms of both weight gain and cytokine production. With respect to organ size, female mice had bigger thymuses and spleens than those of males in both ZS and deficient diet groups.

The amount of tumor necrosis factor (TNF) produced by PEC corresponded with the amount of endotoxin used for stimulation with a maximum production of TNF at the highest dose of endotoxin tested 3 μg/ml. Compared to female mice, ZD male mice produced significantly less TNF. There was no significant difference in TNF production by PECs collected from ZD or ZS female mice. However, if the PECs from ZD female mice were cultured for more than 8 hr, a decreased ability to produce TNF was detected. With regard to the production of interleukin-6 (IL-6), the effect of zinc was more evident. Peritoneal exudate cells from female ZD mice required 10 times the amount of endotoxin to induce IL-6 when compared to PEC from ZS mice. Peritoneal exudate cells from ZD male mice did not produced detectable amounts of IL-6 at any level of endotoxin tested.

These results suggest that even though zinc deficiency does not completely obrogate the ability of PECs to produce cytokines, zinc deficiency can or may downregulate cytokine production and that PECs from male mice are more severely affected than PECs from female mice.

LITERATURE CITED

1. **Abbassy, A. S., M. K. Badr El-Din, A. I. Hassan, G. H. Aref, S. A. Hammad, I. I. El-Araby, A. A. Badr El-Din, M. H. Soliman, and M. Hussein.** 1974. Studies of cell-mediated immunity and allergy in protein energy malnutrition. I. Cell-mediated delayed hypersensitivity. J. Trop. Med. Hyg. **77:**13-17.

2. **Akanji, A. O.** 1990. Malnutrition-related diabetes mellitus in young adult diabetic patients attending a Nigerian diabetic clinic. J. Trop. Med. Hyg. **93:**35-39.

3. **Alvarado, J., and D. G. Luthringer.** 1971. Serum immunoglobulins in edematous protein-calorie malnourished children. Clin. Pediatr. **10:** 174-179.

4. **Anderson, L., and R. S. Weiner.** 1986. Interleukin-1 stimulates fibroblasts to produce granulocyte-macrophage colony stimulating activity and prostaglandin E_2 J. Clin. Invest. **77:**1857-1863.

5. **Bachwich, P. R., S. W. Chensue, J. W. Larrick, and S. L. Kunkel.** 1986. Tumor necrosis factor stimulates interleukin-1 and prostaglandin E_2 production in resting macrophages. **136:**94-101.

6. **Barnes, P.M., and E. J. Moynahan.** 1973. Zinc deficiency in acrodermatitis enteropathica: Multiple dietary intolerance treated with synthetic diet. Proc. R. Soc. Med. **66:** 327-329.

7. **Bashford, C. L., L. Rodrigues, and C. A. Pasternak.** 1989. Protection of cells against membrane damage by haemolytic agents: divalent cations and protons act at the extracellular side of the plasma membrane. Biochimica et Biophysica Acta. **983:**56-64.

8. **Beach, R. S., M. E. Gershwin, and L. S. Hurley.** 1982. Gestational zinc deprivation in mice: Persistence of immunodeficiency for three generations. Science. **218:**469-471.

9. **Beach, R. S., M. E. Gershwin, and L. S. Hurley.** 1980. Growth and development of postnatally zinc-deprived mice. J. Nutr. **110:**201-211.

10. **Bergmann, K. E., E. Makosch, and K. H. Tews.** 1980. Abnormalities of hair zinc concentration in mothers of newborn infants with spina bifida. Am. J. Clin. Nutr. **33:**2145-2150.

11. **Bernard, M. A., and J. L. Rombeau.** 1986. Nutritional support for the elderly patient. In Nutrition, Aging, And Health. E. A. Young, ed. Alan R. Liss, Inc. New York. pp229-259.

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

13. **Beutler, B., and A. Cerami.** 1987. Cachectin: more than a tumor necrosis factor. N. Engl. J. Med. **316:**379-385.

14. **Beutler, B., N. Krochin, I. W. Milsark, C. Luedke, and A. Cerami.** 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. Science. **232:**977-980.

15. **Boosman, R., T. A. Neiwold, C. W. A. A. M. Mutsaers, and E. Gruys.** 1989. Serum amyloid A concentrations in cows given endotoxin as an acute-phase stimulant. Am. J. Vet. Res. **50:**1690-1694.

16. **Brewer, G. J.** 1980. Calmodulin, zinc, and calcium in cellular and membrane regulation: An interpretive review. Am. J. Hemat. **8:**231-248.

17. **Brewer, G. J., V. Yuzbasiyan-Gurkan, D. Y. Lee, and H. Appelman.** 1989. Treatment of Wilson's disease with zinc, VI, initial treatment studies. J. Lab. Clin. Med. **114:**633-638.

18. **Brown, E. R.** 1977. STARVING CHILDREN: The Tyranny of Hunger. Springer Publishing Co. New York.

19. **Businco, L., A. M. Menghi, P. Rossi, R. D'Amelio, and E. Galli.** 1980. Zinc-dependent chemotatic defect in an infant with acrodermatitis. Arch. Dis. Childhood. **55:**966-968.

20. **Carlson, A. B., and T. M. Wardlaw.** 1990. A global, regional and country assessment of child malnutrition. UNICEF, New York.

21. **Çavdar, A. O., A. Arcasoy, T. Baycu, and O. Himmetoglu.** 1980. Zinc deficiency and anencephaly in Turkey. Teratology **22:**141.

22. **Chandra, K. R.** 1984. Nutrition-immunity-infection interactions in old age. In Nutrition, Immunity and Illness in the Elderly. K. R. Chandra, ed. Pergamon Press Inc. New York. pp87-96.

23. **Chandra, R. K.** 1972. Immunocompetence in undernutrition. J. Pediatr. **81:**1194-1200.

24. **Chandra, R. K.** 1974. Rosette-forming T lymphocytes and cell-mediated immunity in malnutrition. Br. Med. J. **3:**608-609.

25. **Chandra, R.K.** 1983. Nutrition, immunity, and infection: present knowledge and future directions. Lancet. 688-691.

26. **Chandra, R.K.** 1983. The nutrition-immunity-infection nexus: the enumeration and functional assessment of lymphocyte subsets in nutritional deficiency. Nutr. Res. **3:**605-615.

27. **Chowdhury, B.A., and R. K. Chandra.** 1986. Trace elemet regulation of immunity and infection. Front. Gastrointest. Res. **13:**134-147.

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

29. **Collart, M. A., D. Berlin, J. D. Vassali, S. deKossodo, and P. Vassali.** 1986. Gamma-interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1 and urokinasegenes, which are controlled by short-lived repressors. J. Exp. Med. **164:**2113-2118.

30. **Coovadia, H. M., M. A. Parent, W. E. K. Loening, A. Wesley, B. Burgess, F. Hallet, P. Brain, J. Grace, J. Naidoo, P. M. Smythe, and G. H. Vos.** 1974. An evaluation of factors associated with the depression of immunity in malnutrition and in measles. Am. J. Clin. Nutr. **27:**665-669.

31. **Coppen, D. E., D. E. Richardson, and R. J. Cousins.** 1988. Zinc Suppression of free radicals induced in cultures of rat hepatocytes by iron, t-butyl hydroperoxide, and 3-methylindole. Proc. Soc. Exp. Biol. Med. **189:**100-109.

32. **Cousins, R. J.** 1979. Regulatory aspects of zinc metabolism in liver and intestine. Nutr. Rev. **37:**97-103.

33. **Cousins, R. J.** 1985. Absorption, transport, and hepatic metabolism of copper and zinc; special reference to metalloenzyme and ceruloplasmin. Physiol. Rev. **65:**238-309.

34. **Cousins, R. J., M. A. Dunn, A. S. Leinart, K. C. Yedinak, and R. A. Disilvestro.** 1986. Coordinate regulation of zinc metabolism and metallothionein gene expression in rats. Am. J. Physiol. **251:**E688-E694.

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

36. **Crawford, I. L., and J. D. Connor.** 1973. Localization and release of glutamic acid in relation to the hippocampal mossy fibre pathway. Nature. **244:**442-443.

37. **Davies, N. T.** 1980. Studies in the absorption of zinc by rat intestine. Br. J. Nutr. **43:**189-203.

38. **Dayer, J. M., B. M. Beutler, A. C. Cerami.** 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblast. J. Exp. Med. **162:**2163-2168.

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

40. **Dreoti, I. E., S. Tao, and L. S. Hurley.** 1968. Plasma zinc and leukocyte changes in weanling and pregnant rats during zinc deficiency. Proc. Soc. Exp. Biol. Med. **128:**169-174.

41. **Dreosti, I. E.** 1983. Zinc and the central nervous system. In Neurobiology of the Trace Elements. I. E. Dreosti, and R. M. Smith, eds. Human Press. New Jersey. pp 135-162.

42. **Dreosti, I. E., and L. S. Hurley.** 1975. Depressed thymidine kinase activity in zinc deficient rat embryos. Proc. Soc. Exp. Biol. Med. **150:**161-165.

43. **Dreosti, I. E., S. J. Manuel, R. A. Buckley, F. J. Fraser, and I. R. Record.** 1981. The effect of late prenatal and/or early postnatal zinc deficiency on the development and some biochemical aspects of the cerebellum and hippocampus in rats. Life Sciences. **28:**2133-2141.

44. **Eaton, J. W., T. D. Skelton, H. S. Swofford, C. E. Kolpin, and H. S. Jacob.** 1973. Elevated erythrocyte calcium in sickle cell disease. Nature **246:**105-106.

45. **Edelman, R., R. Suskind, R. E. Olson, and S. Sirisinha.** 1973. Mechanism of defective delayed cutaneous hypersensitivity in children with protein-calorie malnutrition. Lancet. **1:**506-509.

46. **El-Ghazali, S., M. Mikhail, A. Mohamed, and Shatla, Hamed.** 1990. Significant increase of chromosomal damage in protein energy malnutrition. J. Trop. Medi. Hyg. **93:**372-376.

47. **El-Gholmy, A., S. Hashish, O. Helmy, R. H. Aly, and Y. El-Gamal.** 1970. A study of immunoglobulis in kwashiorkor and marasmus. J. Trop. Med. Hyg. **73:**192-195.

48. **Endres, S., R. Ghorbani, J. G. Cannon, G. Lonnemann, J. W. M. Van Der Meer, S. M. Wolff, and C. A. Dinarello.** 1988. Humans taking dietary omega-3 fatty acids have decreased in vitro production of interleukin-1. In Monokines and Other Non-Lymphocytic Cytokines. Powanda, M. C., J. J. Oppenheim, M. J. Kluger, and C. A. Dinarello eds. Liss Inc. New York. pp153-158.

49. **Fraker, P. J., C. M. Zwickl, and R. W. Luecke.** 1982. Delayed type hyper-sensitivity in zinc deficient adult mice: Impairment and restoration of responsivity to dinitrofluorobenzene. J. Nutr. **112:**309-313.

50. **Fraker, P. J., M. E. Gershwin, R. A. Good, and A. Parasad.** 1986. Interrelationships between zinc and immune function. Fed. Proc. **45:**1474-1479.

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

52. **Ferguson, A. C., G. J. Lawlor, C. G. Neumann, W. Oh, and E. R. Stiehm.** 1974. Decreased rosette-forming lymphocytes in malnutrition and intrauterine growth retardation. J. Pediatr. **85:**717-723.

53. **Ferry, F., and M. Donner.** 1984. In vitro modulation of murine natural killer cytotoxicity by zinc. Scand. J. Immunol. **19:**435-445.

54. **Fields, M., and G. C.Lewis.** 1990. Alcohol consumption aggravates copper deficiency. Metabolism. **39:**610-613.

55. **Flagstad, T.** 1976. Lethal trait A 46 in cattle: Intestinal zinc absorption. Nord Vet. Med. **28:** 160-169.

56. **Fleming, C. R., L. M.Smith, and R. E. Hodges.** 1976. Essential fatty acid deficiency in adults receiving total parenteral nutrition. Am. J. Clin. Nutr. **29:**976-983.

57. **Flynn, A., M. A. Loftus, and J. H. Finke.** 1984. Production of interleukin-1 and interleukin-2 in allogeneic mixed lymphocyte cultures under copper, magnesium and zinc deficient conditions. Nutr. Res. **4:**673-679.

58. **Foote, J. W., and H. T. Delves.** 1984. Albumin bound and α_2-bound zinc concentrations in the sera of healthy adults. J. Clin. Pathol. **37:**1050-1054.

59. **Goldberg, A. L., V. Baracos, P. Rodemann, L. Waxman, and C. A. Dinarello.** 1984. Control of protein degradation in muscle protein by prostaglandins, Ca^{2+}, and leukocytic pyrogen (interleukin1). Fed. Proc. **43:** 1301-1306.

60. **Goldblum, S. E., D. A. Cohen, M. Jay, and C. J. McClain.** 1987. Interleukin 1-induced depression of iron and zinc: role of granulocytes and lactoferrin. Am. J. Physiol. **252:**E27-E32.

61. **Good, R. A., A. West, N. K. Day, Z. W. Dong, and G. Fernandes.** 1982. Effects of undernutrition on host cell and organ function. Cancer Res. **42:**737S-743S.

62. **Gresser, I.** 1977. On the varied biological effects of interferon. Cell. Immunol. **34:**406-415.

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

64. **Hambidge, K. M., P. A.Walravens, R. M. Brown, J. Webster, S. White, M. Anthony, and M. L. Roth.** 1976. Zinc nutrition of preschool children in the Denver Head Start Program. Am. J. Clin. Nutr. **29:**734-738.

65. **Hambidge, K. M., C. Hambidge, M. Jacobs, and J. D. Baum.** 1972. Low levels of zinc in hair, anorexia, poor growth, and hypogeusia in children. Pediat. Res. **6:**868-877

66. **Hambidge, K. M., C. E. Casey, and N. F. Krebs.** 1986. Zinc. In Trace Elements In Human And Animal Nutrition. W. Mertz, ed. Academic Press Inc. San Diego. pp1-137.

67. **Hambidge, K. M., K. H. Neldner, and P. A. Walravens.** 1975. Zinc, acrodermatitis enteropathica and congenital malformations. Lancet. **1:**577-578.

68. **Hendricks, K.M., and W. A. Walker.** 1988. Zinc deficiency in inflammatory bowel disease. Nutrition Review **46:**401-408.

69. **Hunt, D. C., P. E. Johnson, J. Herbal, and L. K. Mullen.** 1992. Effects of dietary zinc depletion on seminal volume and zinc loss, and serum testosterone concentrations, and sperm morphology in young men. Am. J. Clin. Nutr. **56:**148-157.

70. **Hurley, L. S., and H. Swenerton.** 1966. Congenital malformations resulting from zinc deficiency in rats. Proc. Soc. Exp. Biol. Med. **123:**692-696.

71. **Hurley, L. S., and R. E. Shrader.** 1975. Abnormal development of preimplantation rat eggs after three days of maternal zinc deficiency. Nature. **254:**427-429.

72. **Hutt, M. S. R.** 1969. Malnutrition and infection-East African studies. J. Trop. Pediatr. **15:**152-154.

73. **Iwata, T., G. S. Incefy, T. Tanaka, G. Fernendes, C. J. Menendez-Botet, K. Pih, and R. A. Good.** 1979. Circulating thymic hormone levels in zinc deficiency. Cell. Immunol. **47:**100-105.

74. **James, S. J., M. Swendseid, and T. Makinodan.** 1987. Macrophage-mediated depression of T-cell proliferation in zinc-deficient mice. J. Nutr. **117:**1982-1988.

75. **Jameson, S.** 1976. Effects of zinc deficiency in human reproduction. Acta. Med. Scand. 200:Suppl **593:**3-89.

76. **Keet, M. P., and H. Thom.** 1969. Serum immunoglobulins in kwashioorkor. Arch. Dis. Child. **44:**600-603.

77. **Keilin, D., and J. Mann.** 1940. Carbonic anhydrase. Purification and nature of the enzyme. Biochem. J. **34:**1163-1177.

78. **Kernkamp H.C.H., and E. F. Ferrin.** 1953. Paraketosis in swine. JAVMA. **123:**217-220.

79. **Kettlehut, I. C., W. Fiers, and A. L. Goldberg.** 1987. Toxic effects of tumor necrosis factor in vivo and their prevention by cyclooxygenase inhibitors. Proc. Natl. Acad. Sci. USA. **84:**4273-4277.

80. **Kimura, A., and B. Ersson.** 1981. Activation of T lymphocytes by lectins and carbohydrate-oxidising reagents viewed as an immunological recognition of cell-surface modifications seen in the context of "self" major histocompatibility complex antigens. Eur. J. Immunol. **11:**475-483.

81. **Klasing, K. C.** 1988. Nutritional aspects of leukocytic cytokines. J. Nutr. **118:**1436-1446.

82. **Klasing, K. C., D. E. Laurin, R. K. Peng, and D. M. Fry.** 1987. Immunologically mediated growth depression in chicks: Influence of feed intake, corticosterone and interleukin-1. J. Nutr. **117**:1629-1637.

83. **Kunkel, S. L., M. Spengler, M. M. May, R. Spengler, J. Larrick, and D. Remick. 1988.** Prostaglandin E_2 regulates macrophage-derived tumor necrosis factor gene expression. J. Biol. Chem. **263**:5380-5384.

84. **Laurence, K. M., N. James, M. Miller, and H. Campbell.** 1980. Increased risk of recurrence of pregnancies complicated by fetal neural tube defects in mothers receiving poor diets and possible benefit of dietary counselling. Br. Med. J. **281**:1592-1594.

85. **Le, J., and J. Vileek. 1987.** Tumor necrosis factor and interleukin-1: Cytokines with multiple overlapping biological activities. Lab. Invest. **56**: 234-248.

86. **Leucke, R. W., C. E. Simonel, and P. J. Fraker.** 1978. The effect of restricted diet intake on the antibody mediated response of the zinc deficient A/J mouse. J. Nutr. **108**:881- 887.

87. **Mathan, V. I.** 1988. Tropical sprue in southern india. Trans. R. Soci. Trop. Medi. Hyg. **82:** 10-14.

88. **McCaffrey, R., D. F. Smoler, and D. Baltimore.** 1973. Terminal deoxyribo-nucleotidyl transferace in a case of childhood lymphoblastic leukemia. Proc. Natl. Acad. Sci. USA. **70**:521-525.

89. **McFarlane, H., S. Reddy, K. J. Adcock, H. Adeshina, A. R. Cooke, and J. Akene.** 1970. Immunity, transferrin and survival in kwashiorkor. Br. Med. J. **4**:268-270.

90. **Michael, G.** 1988. The effects of malnutrition in the metabolism of children. Trans. R. Soci. Trop. Medi. Hyg. **82**:3-6.

91. **Moldawer, L. L., and S. F. Lowry.** 1988. Cachectin: Its impact on metabolism and nutritional status. Ann. Rev. Nutr. **8**:585-609.

92. **Nishi, Y., F. L. Lifshitz, M. A. Bayne, F. Daum, M. Silverberg, and H. Aiges.** 1980. Zinc status and its relation to growth retardation in children with chronic inflammatory bowel disease. Am. J. Clin. Nutr. **33**:2613-2621.

93. **O'Dell, B. L., and J. E. Savage.** 1960. Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med. **103**:304-306.

94. **Otsuka, F., and M. Ohsawa.** 1991. Differential susceptibility of T- and B-lymphocyte proliferation to cadmium: Relevance to zinc requirement in T-lymphocyte proliferation.

95. **Otvos, J. D., D. H. Petering, and C. F. Shaw.** 1989. Structure-reactivity relationships of metallothionein, a unique metal-binding protein. Comments. Inorg. Chem. **9**:1-35.

96. **Pencharz, P. B., M. Azcue, and N. Vaisman.** 1992. Nutritional implications for the treatment of anorexia nervosa and bulimia nervosa. In The Biology of Feast and Famine: Relevance to Eating Disorders. Anderson, G. H., and S. H. Kennedy, eds. Academic Press Inc. San Diego. pp357-367.

97. **Piletz, Z.E., and R. E. Ganshow.** 1978. Zinc deficiency in murine milk underlies expression of the lethal milk (lm) mutation. Science. **199**:181-183.

98. **Prasad, A. S.** 1991. Discovery of human zinc deficiency and studies in an experimental human model. Am. J. Nutr. **53**:403-412.

99. **Prasad, A. S., J. A. Halsted, and M. Nadimi.** 1961. Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. Am. J. Med. **31**:532-546.

100. **Prasad, A. S., A. Miale Jr., Z. Farid, H. H. Sandstead, and W. J. Darby.** 1963. Biochemical studies on dwarfism, hypogonadism, and anemia. Arch. Intern. Med. **111:** 407-428.

101. **Prasad, A. S., and D. Obserleas.** 1974. Thymidine kinase activity and incorporation of thymidine into DNA in zinc deficient tissue. J. Lab. Clin. Med. **83**:634-639.

102. **Prasad, A. S., S. Meftah, J. Abdallah, J. Kaplan, G. J. Brewer, J. F. Bach, and M. Dardenne.** 1988. Serum thymulin in human zinc deficiency. J. Clin. Invest. **82**:1202-1210.

103. **Raulin, J.** 1869. Etudes chimique sur la vegetation. Ann. Sci. Nalt. Botan. Biol. Vegetale. **11**:93-99.

104. **Reardon, C. L., and D. O. Lucas.** 1987. Heavy metal mitogenesis: Thymocyte activation by Zn^{++} requires 2-mercaptoethanol and lipopoly-saccharide as cofactors. Immunobiol. **174**:233-243.

105. **Reardon, C. L., and D. O. Lucas.** 1987. Heavy metal mitogenesis: Zand Hg^{++} induce cellular cytotoxicity anf interferon production in murine T lymphocytes. Immunobiol. **175:**455-469.

106. **Richard, C. B.** 1976. Copper, zinc, and manganese content of four amino acid and protein hydrolysate preparations. Am. J. Clin. Nutr. **29:**1331-1332.

107. **Robbins, L. S., and V. Kumar.** 1987. Nutritional Disorders. In Basic Pathology 4th ed. W. B. Saunders com. Philadelphia. pp235-252.

108. **Rosen, F. S.** 1971. The complement system and increased susceptibility to infection. Semin. Hematol. **8:**221-226.

109. **Saba, T. M., and N. R. DiLuzio.** 1968. Involvement of the opsonic system in starvation induced depression of the reticuloendothelial syatem. Proc. Soc. Exp. Biol. Med. **128:**869-875.

110. **Sandstead, H. H.** 1973. Zinc nutrition in the United States. Am. J. Clin. Nutr. **26:**1251-1256.

111. **Schlesinger, L., A. Ohlbaum, L. Grez, and A. Stekel.** 1976. Decreased interferon production by leukocytes in marasmus. Am. J. Clin. Nutr. **29:**758-761.

112. **Schlesinger, L., and A. Stekel.** 1974. Impaired cellular immunity in marasmic infants. Am. J. Clin. Nutr. **27:**615-620.

113. **Schroeder, J. J., and R. Cousins.** 1990. Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proc. Natl. Acad. Sci. USA. **87:**3137-3141.

114. **Scuderi, P.** 1989. Differential effects of copper and zinc on human peripheral blood monocyte cytokine secretion. Cell. Immunol. **126:**391-405.

115. **Smythe, P. M., M. Schonland, G. G. Brereton-Stiles, H. M. Coovadia, H. J. Grace, W. E. K. Loening, A. Mafoyane, M. A. Parent, and G. H. Vos.** 1971. Thymolymphatic deficiency and depression of cell-mediated immunity in protein-calorie malnutrition. Lancet. **2:**939-944.

116. **Snyder, S.L., and R. I. Walker.** 1976. Inhibition of lethality in endotoxin-challenged mice treated with zinc chloride. Infect. Immun. **13:**998-1000.

117. **Snyder, D. S., and E. R. Unanue.** 1982. Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. J. Immunol. **129:**1803-1805.

118. **Sobocinski, P. Z., M. C. Powanda, W. J. Canterbury, S. V. Machotka, R. I. Walker,and S.L. Snyder.** 1977. Role of zinc in the abatement of hepatocellular damage and mortality induced in endotoxemic rats. Infect. immun. **15:**950-957.

119. **Strieter, R. M., D. G. Remick, P. A. Ward, R. N. Splengler, J. P. Lynch, III, J. Larrick, and S. L. Kunkel.** 1988. Cellular and molecular regulation of tumor necrosis factor-alpha production by pentoxifylline. Biochem. Biophys. Res. Commun. **155:**1230-1236.

120. **Suskind, R., S. Sirishinha, V. Vithayasai, R. Edelman, D. Damrongsak, C. Charupatana, and R. E. Olson.** 1976. Immunoglobulins and antibody response in children with protein calorie malnutrition. Am. J. Clin. Nutr. **29:** 836-841.

121. **Swenerton, H., R. Shrader, and L. S. Hurley.** 1969. Zinc deficienct embryos: reduced thymidine incorporations. Science. **166:**1014-1015.

122. **Swerdel, M. R., and R. J. Cousins.** 1982. Induction of kidney metal-lothionein and metallothionein messenger RNA by zinc and cadmium. J. Nutr. **112:**801-809.

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

124. **Tite, J. P., G. Dougan, and S. N. Chatfield.** 1991. The involvement of tumor necrosis factor in immunity to *Salmonella* infection. J. Immunol. **147:**3161-3164.

125. **Tobias, P. S., K. P. W. J. MacAdam, and R. J. Ulevitch.** 1982. Interactions of bacterial lipopolysaccharide with acute-phase rabbit serum and isolation of two forms of rabbit serum amyloid A. J. Immunol. **128:**1420-1427.

126. **Tracey, K. J., S. F. Lowry, T. J. Fashey,III, J. D. Albert, Y. Fong, D. Hesse, B. Beutler, K. R. Monogue, S. Calvano, H. Wei, A. Cerami, and T. Shires.** 1987. Cachectin/tumor necrosis factor induces lethal septic shock and stress hormone responses in the dog. Surg. Gynecol. Obstet. **164:**415-422.

127. **Tucker H. F., and W. D. Salmon.** 1955. Paraketosis or zinc deficiency disease in the pig. Proc. Soc. Exp. Biol. Med. **88:** 613-616.

128. **Uehara, A., P. E. Gottschall, R. R. Dahl, and A. Arimura.** 1987. Stimulation of ACTH release by human interleukin-β, but not by interleukin-α, in conscious, freely-moving rats. Biochem. Biophys. Res. Commun. **146:**1286-1290.

129. **Vruwink, K. G., M. P. Fletcher, C. L. Keen, M. S. Golub, A. G. Hendrickx, and M. E. Gershwin.** 1991. Moderate zinc deficiency in rhesus monkeys. An intrinsic defect of neutrophil chemotaxix correlated by zinc repletion. J. Immunol. **146:**244-249.

130. **Warner, G. L., and D. A. Lawrence.** 1986. Stimulation of murine lymphocyte responses by cations. Cell. Immunol. **101:**425-439.

131. **Warren, R. S., H. F. Starnes, Jr., N. Alcock, S. Calvano, and M. F. Brennan.** 1988. Hormonal and metabolic response to recombinant human tumor necrosis factor in rat: in vitro and in vivo. Am. J. Physiol. **255:**E206-E212.

132. **Warren, R. S., H. F. Starnes, Jr., J. L. Gabrilove, H. F. Oettgen, and M. F. Brennan.** 1987. The acute metabolic effects of tumor necrosis factor administration in humans. Arch. Surg. **122:**1396-1400.

133. **Watts, T.** 1969. Thymus weights in malnourished children. J. Trop. Pediatr. **15:**155-158.

134. **Winchurch, R. A.** 1988. Activation of thymocyte response to interleukin-1 by zinc. Clin. Immunol. Immunopath. **47:**174-180.

135. **Work, T. H., A. Ifekwunik, D. B. Jelliffe, P. Jelliffe, and C. G. Neumann.** 1973. Tropical problems in nutrition. Ann. Intern. Med. **79:**701-711.

136. **Yang, R. S., L. L. Moldawer, A. Sakamoto, R. A. Keenan, D. E. Matthews, V. R. Young, R. W. Wannemacher, Jr., G. L. Blackburn, and B. R. Bistrian.** 1983. Leukocyte endogenous mediator alters protein dynamics in rats. Metabolism **32:**654-660.

137. **Zhang, Y., J. X. Lin, Y. K. Yip, and J. Vileek.** 1988. Enhancement of cAMP levels and of protein kinase activity by tumor necrosis factor and interleukin 1 in human fibroblasts: Role in the induction of interleukin 6. Proc. Natl. Acad. Sci. USA. **85:**6802-6805.

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

ACKNOWLEDGEMENTS

So many people have helped and provided me with love and support in the completion of this thesis. First of all, I would like to thank my major professor, Dr. Michael J. Wannemuehler, for the direction and invaluable help during this work. Without his patience and encouragement, this work would not be done. I would also like to thank Drs. Richardo F. Rosenbusch and Malcolm H. Crump for serving on my program of study committee and help in the critical review this thesis.

I would like to express my thanks to my friends. Firstly, I would like to thank Linda L. Ritland for her great technical help and friendship. I would also like to thank my Lab family for their care and encouragement during the work. Special thanks are extended to my fellow Korean friends Im-Seok Kang, Hyun-Dong Paik, and Sang-Gu Kang and their families for their encouragement and sincere friendship. Other friends within VMRI, MIPM, and I.S.U. have also helped me with their invaluable efforts.

I would also like to express my appreciation to Zinpro, Inc., for their financial support of the zinc project on which I worked.

I would like to say thank you very much to my mother for her endless financial and emotional support. I would like to thank my brothers and family in laws for their ceaseless help.

Lastly, I would thank my wife Sung-Ok, and two daughters, Sang-Hwa and Rachael Hae-Gi, for their everlasting love and endurance during this work. Without your love, I could not overcome hard times.

Thank you very much GOD for your grant of life and chance to meet these good people.